

F-ACTIN REGULATION OF SNARE-MEDIATED INSULIN SECRETION

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DEDICATION

I dedicate this dissertation to my parents Adam Kalwat and Karen Ley, my grandparents Roland and Sandy Ley and Helen Kalwat, and my wife Danielle. Without their love and support I would never have reached this point.

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ABSTRACT

Michael Andrew Kalwat

F-ACTIN REGULATION OF SNARE-MEDIATED INSULIN SECRETION

In response to glucose, pancreatic islet beta cells secrete insulin in a biphasic manner, and both phases are diminished in type 2 diabetes. In beta cells, cortical F-actin beneath the plasma membrane (PM) prevents insulin granule access to the PM and glucose stimulates remodeling of this cortical F-actin to allow trafficking of insulin granules to the PM. Glucose stimulation activates the small GTPase Cdc42, which then activates p21-activated kinase 1 (PAK1); both Cdc42 and PAK1 are required for insulin secretion. In conjunction with Cdc42-PAK1 signaling, the SNARE protein Syntaxin 4 dissociates from F-actin to allow SNARE complex formation and insulin exocytosis. My *central hypothesis* is that, in the pancreatic beta cell, glucose signals through a Cdc42-PAK1-mediated pathway to remodel the F-actin cytoskeleton to mobilize insulin granules to SNARE docking sites at the PM to evoke glucose stimulated second phase insulin secretion. To investigate this, PAK1 was inhibited in MIN6 beta cells with IPA3 followed by live-cell imaging of F-actin remodeling using the F-actin probe, Lifeact-GFP. PAK1 inhibition prevented normal glucose-induced F-actin remodeling. PAK1 inhibition also prevented insulin granule accumulation at the PM in response to glucose. The ERK pathway was implicated, as glucose-stimulated ERK activation was decreased under PAK1-depleted conditions. Further study showed that inhibition of ERK impaired insulin secretion and cortical F-actin remodeling. One of the final steps of insulin secretion is the fusion of insulin granules with the PM which is facilitated by the SNARE proteins Syntaxin 4 on the PM and VAMP2 on the insulin granule. PAK1 activation was also found to be critical for Syntaxin 4-F-actin complex dynamics in beta cells, linking the Cdc42-PAK1 signaling pathway to SNARE-mediated exocytosis. Syntaxin 4 interacts with the F-actin severing protein Gelsolin, and in response to

glucose Gelsolin dissociates from Syntaxin 4 in a calcium-dependent manner to allow Syntaxin 4 activation. Disrupting the interaction between Syntaxin 4 and Gelsolin aberrantly activates endogenous Syntaxin 4, elevating basal insulin secretion. Taken together, these results illustrate that signaling to F-actin remodeling is important for insulin secretion and that F-actin and its binding proteins can impact the final steps of insulin secretion.

Debbie C. Thurmond, PhD, Chair

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LIST OF ABBREVIATIONS

[Ca ²⁺] _c	Cytosolic Calcium Concentration
ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
cAMP	Cyclic AMP
Cav1	Caveolin 1
Cdc42	Cell Division Cycle 42
CHO-K1	Chinese Hamster Ovary Cell Line
CRIB	Cdc42/Rac Interactive Binding
DMSO	Dimethyl Sulfoxide
Epac2	Exchange Protein Directly Activated by cAMP 2
ERK	Extracellularly-Regulated Kinase
F-actin	Filamentous Actin
FAK	Focal Adhesion Kinase
G-actin	Globular Actin
GAP	Guanosine Triphosphatase Activating Protein
GDI	Guanosine Triphosphate Dissociation Inhibitor
GDP	Guanosine Diphosphate
GEF	Guanosine Exchange Factor
GFP	Green Fluorescent Protein
GLUT2	Glucose Transporter 2
GSIS	Glucose-Stimulated Insulin Secretion
GST	Glutathione S-Transferase
GTP	Guanosine Triphosphate
GTPase	Guanosine Triphosphatase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
INS 832/13	Rat Insulinoma 832/13 Cell Line
K _{ATP}	ATP-sensitive Potassium Channel
KRBH	Krebs-Ringer Bicarbonate HEPES Buffer
MEK	MAPK/ERK kinase
MIN6	Mouse Insulinoma 6 Cell Line
MKRBB	Modified Krebs-Ringer Bicarbonate Buffer
MLC2	Myosin Light Chain 2
MLCK	Myosin Light Chain Kinase
MODY	Maturity-Onset Diabetes of Youth
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NP-40	Nonidet P-40 Detergent
NSF	N-Ethylmaleimide Sensitive Factor
N-WASP	Neuronal Wiskott-Aldrich Syndrome Protein
PAK1	p21-Activated Kinase 1
PBS	Phosphate Buffered Saline
PC12	Phenochromocytoma 12 Cell Line
PIP	phosphatidylinositol 4-phosphate
PIP2	phosphatidylinositol 4,5-bisphosphate
PM	Plasma Membrane
Rac1	Ras-Related C3 Botulinum Toxin Substrate 1
Rho	Ras Homologous
RRP	Readily Releasable Pool
SDS-PAGE	Sodium-Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SM	nSec1/Munc18
SNAP-25	Synaptosomal-Associated Protein 25
SNARE	Soluble NSF-Attachment Protein Receptor
t-SNARE	target-SNARE
VAMP2	Vesicle-Associated Membrane Protein 2
VDCC	Voltage-Dependent Calcium Channel
v-SNARE	vesicle-SNARE

CHAPTER 1. INTRODUCTION

1.1 GLUCOSE HOMEOSTASIS AND TYPE 2 DIABETES

The disease diabetes has been known for more than two millennia, but most of the advancements in our knowledge of the etiology of the disease have come about in the last 150 years (3). Diabetes mellitus literally means ‘sweet urine’ and is so named because individuals with advanced uncontrolled disease have hyperglycemia that leads to glucose excretion into the urine (3). The maintenance of blood glucose homeostasis is critical for survival, and is controlled by the coordination of multiple organs and endocrine systems. Upon consumption of a meal, blood glucose levels increase and pancreatic islet beta cells sense this increase and secrete the hormone insulin in response. Insulin suppresses hepatic glucose production and signals to skeletal muscle and adipose tissue to take up the excess blood glucose. This process maintains the blood glucose concentration within a range of about 110-125 mg/dl (5.6-6.9 mM) in humans (4). If both the secretion of insulin and the uptake of glucose by peripheral tissues fail, the resulting hyperglycemia leads to type 2 diabetes mellitus (3).

As of 2011, about 8% of the United States population has been diagnosed with diabetes (5). In adults, type 2 diabetes accounts for 90-95% of all diabetes cases, while type 1 diabetes accounts for about 5% (5). Distinct from type 2 diabetes, type 1 diabetes occurs when the body's immune system attacks the pancreatic beta cells causing a loss of beta cell mass and a loss of insulin secretion. Accounting for the remaining percentage are cases of maturity-onset diabetes of youth and gestational diabetes. Maturity-onset diabetes of youth is caused by heritable genetic mutations that account for 1-5% of all diabetes cases (5). Gestational diabetes occurs in up to 18% of pregnant women, of those women, 5% to 10% of women will develop type 2 diabetes immediately after pregnancy, and the remaining 8-13% will have ~ 60% chance of developing diabetes in the following 10-20 years (5). Insulin resistance in the liver can also contribute to hyperglycemia in diabetes due to a failure to suppress hepatic glucose output (6).

Current research suggests that in response to peripheral insulin resistance and elevated blood glucose levels, beta cells compensate by increasing mass, resulting in increased insulin production (7). After years of compensation, the beta cells eventually die, resulting in diabetes (8). Some individuals become obese and compensate with beta cell expansion but never progress to type 2 diabetes, indicating some environmental and/or epigenetic contribution to the disease (9). Concomitant with beta cell loss in type 2 diabetes, there is support for beta cell dysfunction since islets from type 2 diabetic donors have impaired glucose-stimulated insulin secretion (GSIS) compared to normal donor islets but without a significant difference in islet insulin content (10, 11). This notion drives research in the field toward therapeutics that may increase the function of these existing beta cells. Other avenues of research are focused on preventing beta cell death, enhancing beta cell regeneration, or development of stem cell and islet transplantation therapies to replace lost beta cells (12-14). Furthermore, many of the genes identified in human genome-wide association studies of diabetic individuals are directly associated with beta cell function, cementing the theory that beta cell dysfunction is central to the development of type 2 diabetes (15, 16).

1.2 THE PANCREATIC ISLET

Pancreatic islets of Langerhans, first described by Paul Langerhans in 1869, are small clusters of multiple endocrine cell types that comprise about 1-2% of the pancreas by mass (3, 17). Pancreatic islets consist of at least 4 major cell types: insulin secreting beta cells, glucagon secreting alpha cells, somatostatin secreting delta cells, and pancreatic polypeptide secreting PP cells (18). The architecture of human islets is such that alpha and beta cells are mixed throughout the islet (19). Recently, it was discovered in human islets that alpha cells are adjacent to blood vessels while beta cells are more interior and, due to the invaginations of epithelium unique to human islets there are greater numbers of alpha cells in the core of the islet (20). These new

findings suggest rodent islets are actually similar to human islets. Since rodent islets contain a core of beta cells with alpha cells near the periphery, perhaps the difference in overall cell organization is merely due to the vasculature. An islet can contain from a few to 5000 cells with beta cells making up at least two-thirds of the islet (3). Each beta cell contains ~10,000 granules with about 5% of these granules pre-docked at the plasma membrane and about 20% within 300 nm (roughly one granule diameter) from the plasma membrane (21, 22). The large amount of insulin granules gives the beta cell the capacity to secrete insulin over long periods of time. Although the beta cell stores many granules, each with a half-life of ~3-5 days, newly synthesized granules can be secreted as early as 45 min after synthesis and are preferentially secreted over older granules (23-25).

1.2.1 Biphasic Insulin Secretion from Pancreatic Islet Beta Cells

In response to increased blood glucose, pancreatic islet beta cells secrete insulin in a biphasic manner (Figure 1-1). Glucose enters the beta cell and is rapidly metabolized to yield a net increase in the ATP/ADP ratio. As a result, ATP-sensitive potassium (K_{ATP}) channels close and cause membrane depolarization which opens voltage-dependent calcium channels (26). Subsequent calcium influx leads to insulin granule exocytosis (27-29). This stimulus-secretion coupling pathway results in a rapid robust spike of insulin secretion called 'first-phase', derived from insulin granules pre-docked/juxtaposed within 100-200 nm of the plasma membrane, and referred to as the 'readily releasable pool' (RRP) (30, 31). After the first-phase peak, the insulin release rate drops to 2 to 5 fold above basal secretion and is sustained at this lower rate of insulin release, called 'second-phase', which persists until euglycemia is restored (32).

Second-phase insulin secretion thought to require the recruitment of granules from intracellular storage pools to the plasma membrane and involves reorganization of the filamentous actin (F-actin) cytoskeletal network (Figure 1-2A) (33-35). Specifically, the reorganization takes place in the cortical F-actin web (Figure 1-2B). In type 2 diabetes there is a

loss of both first and second phase insulin secretion (36). As insulin secretion can occur over the course of hours, the second phase can account for the majority of insulin released when compared to the first phase, although this does not discount the importance of the first phase in curtailing hepatic glucose output and glucagon secretion from alpha cells (37, 38). There is far less known about the mechanistic regulation of second-phase insulin secretion. Key proteins shown to be required or have a role in both phases of insulin secretion include the SNARE protein Syntaxin 4, the SNARE-related protein tomosyn-2, and granuphilin (39-41). The SNARE protein Syntaxin 1 is only required for first-phase (42). The SNARE proteins will be described in detail in section 1.7. Currently identified regulators specific to second phase insulin secretion include, Cdc42, PAK1, and RhoGDI (43-45). These will be discussed in detail in later sections.

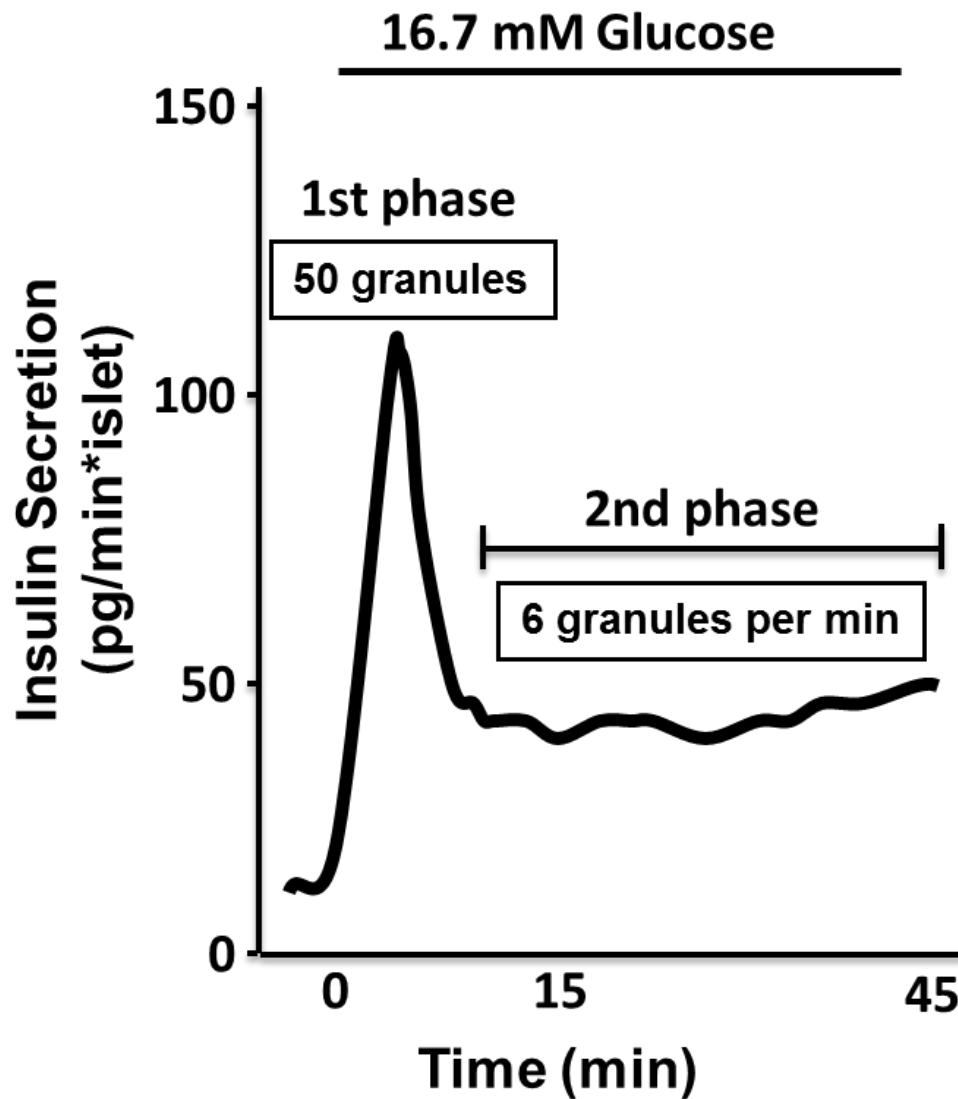


Figure 1-1. Biphasic glucose-stimulated insulin secretion from islet beta cells. In response to a square-wave increase in glucose concentration, islet beta cells secrete about 50 granules in a first phase of secretion. This phase is thought to be accounted for by the RRP granules depicted in the figure. When the glucose stimulus persists, beta cells secrete in a second phase that is a lower rate, sustained process that can continue for hours. Second phase is thought to require the reserve pool granules to be recruited to the plasma membrane for secretion.

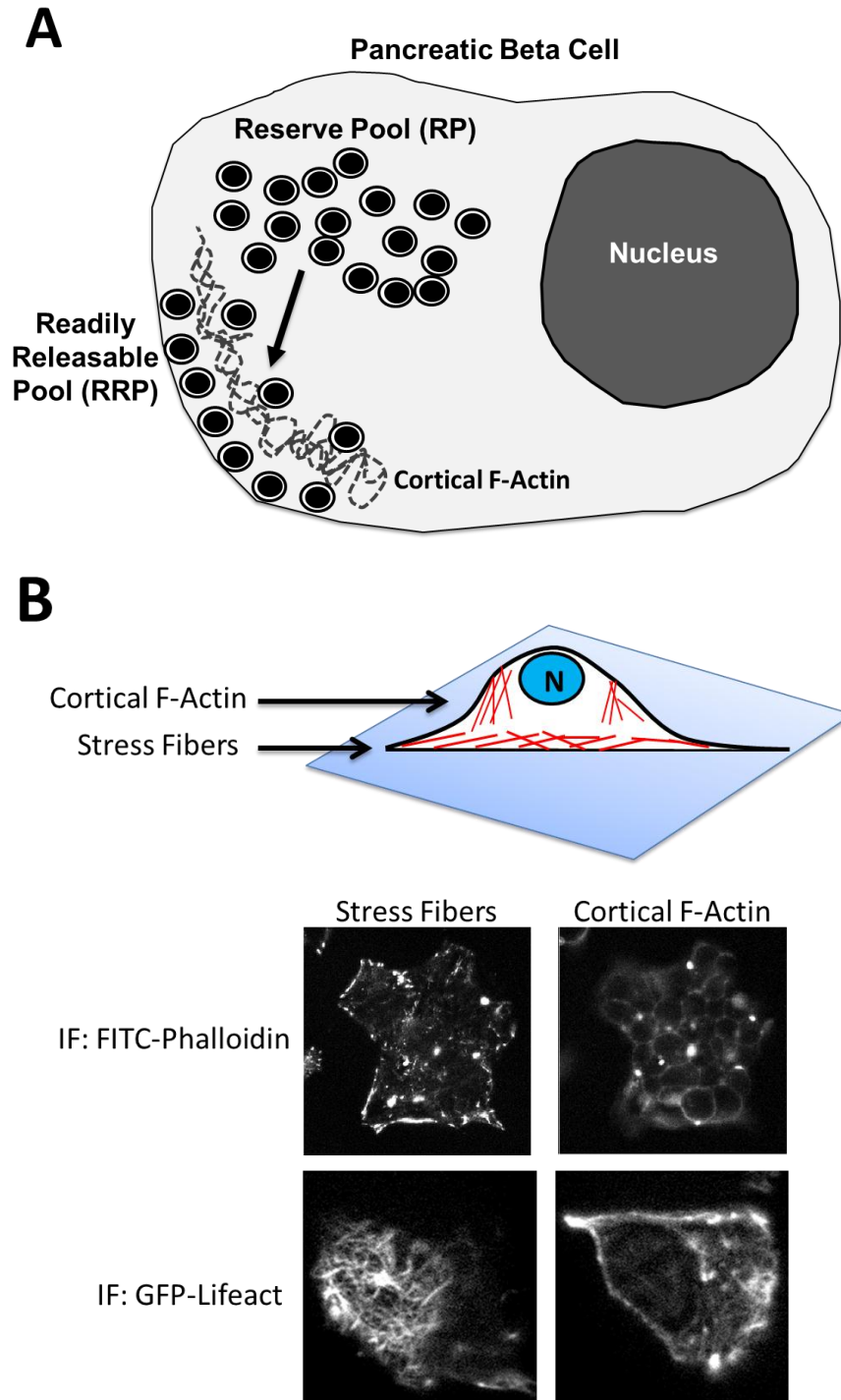


Figure 1-2. F-actin regulates granule access to the readily releasable pool. **A)** Insulin granules are stored in an intracellular reserve pool behind a cortical web of F-actin (1). Granules that are already present at or near (<50nm) the plasma membrane are considered the readily releasable pool (RRP). Upon glucose stimulation, this F-actin is remodeled to allow granules to translocate to the RRP for secretion. **B)** Cartoon showing difference between stress fibers and cortical F-actin just below the plasma membrane at the midplane of the beta cell. Lower panels show confocal images of MIN6 cells either stained with FITC-Phalloidin (MIN6 cell cluster is shown, courtesy of Dr. Angela Nevins) or expressing a live-cell F-actin probe Lifeact-GFP (single cell is shown) to show F-actin localization.

1.2.2 The Triggering and Amplifying Pathways of Insulin Secretion

Initially described 20 years ago by two different research groups, the triggering and amplifying pathways describe how glucose metabolism elicits insulin secretion (46, 47). The ability of glucose to cause an increase in cytosolic $[Ca^{2+}]$ resulting in insulin secretion is referred to as the triggering pathway (2). The triggering pathway (sometimes referred to as the K_{ATP} -dependent pathway) can be activated by other non-nutrient secretagogues such as KCl, which depolarizes the beta cell, causing calcium channels to open and insulin granules pre-docked at the PM to fuse and release their insulin cargo from the beta cell (48). The triggering pathway is commonly associated with first-phase insulin secretion. In addition to the triggering effect, glucose metabolism results in additional signals that enhance the amount of insulin secreted in response to the triggering pathway, termed the amplifying pathway (also referred to as the K_{ATP} -independent pathway) (48). It is important to note that the amplifying pathway requires the initial triggering pathway in order to manifest its effects on insulin secretion. The amplifying pathway is commonly associated with second-phase insulin secretion, although there is evidence now that amplification has effects in both phases of insulin secretion (34, 49). The triggering and amplifying pathways can be separated *in vitro* using a drug called diazoxide. Diazoxide binds to the K_{ATP} channel holding it open even in the presence of elevated ATP levels (Figure 1-3A) (50). Under the diazoxide paradigm, while K_{ATP} channels are held open, depolarizing levels of KCl trigger calcium influx and insulin secretion. Further addition of glucose elicits amplified insulin secretion and is termed the amplifying pathway (Figure 1-3B) (48). Importantly, the amplifying pathway does not cause further elevation of cytosolic $[Ca^{2+}]$ (48). Sulfonylurea drugs can also be used to demonstrate the amplifying pathway by binding to a regulatory subunit of the K_{ATP} channel to cause channel closure which induces membrane depolarization (2). Use of either diazoxide or sulfonylurea essentially clamps the beta cell K_{ATP} channels so that they are not affected by glucose-induced elevations in ATP concentration, and so any changes in insulin secretion in response to glucose are independent of changes in cytosolic $[Ca^{2+}]$. This model

system has proven key towards elucidating the factors and signaling events underlying this otherwise uncharacterized amplifying/second-phase secretory pathway.

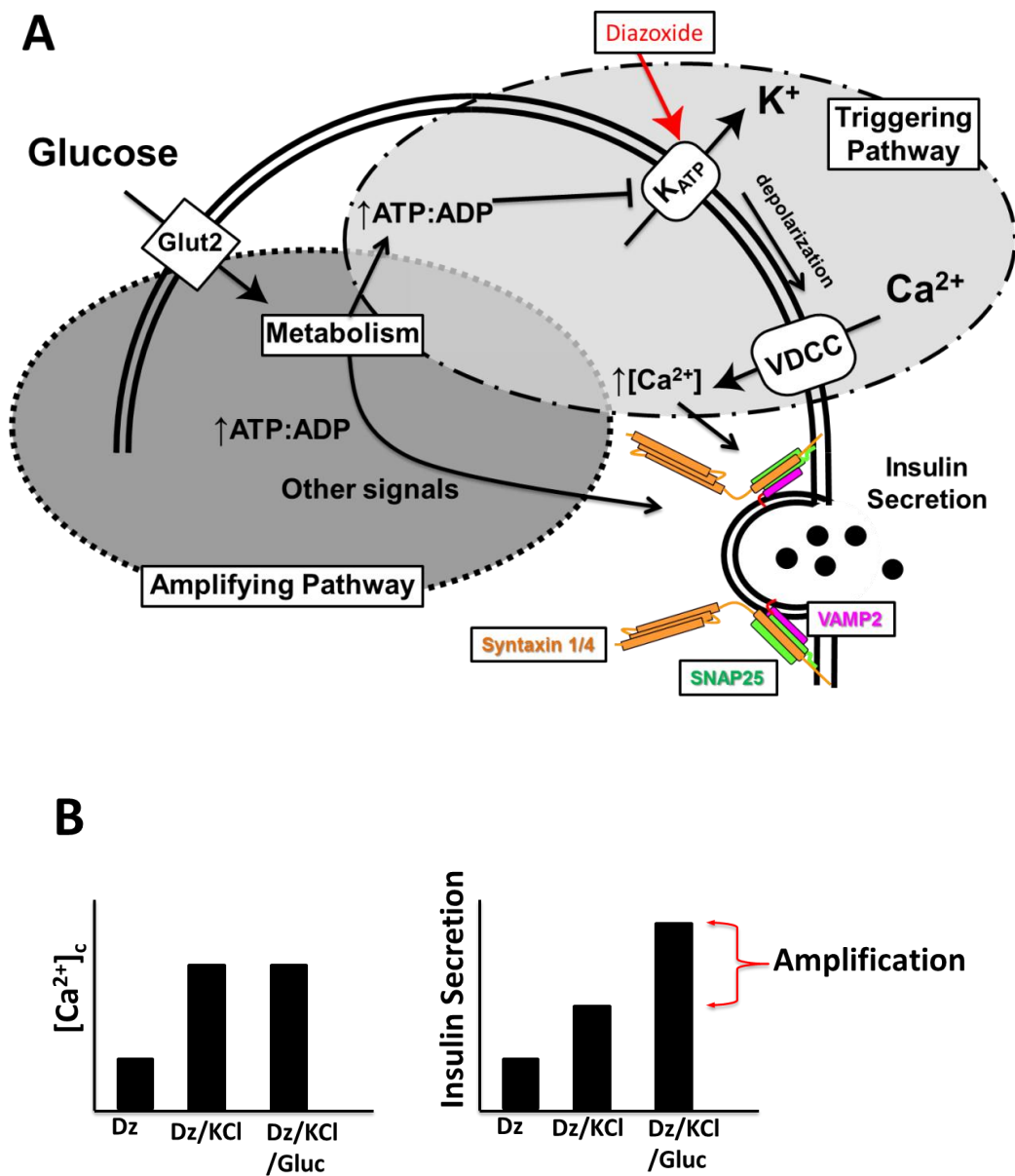


Figure 1-3. The triggering and amplifying pathways of insulin secretion. **A)** Upon entry into the beta cell through the Glut2 transporter, glucose is metabolized leading to increased ATP/ADP ratio which closes ATP-sensitive K⁺ channels (K_{ATP}) to cause membrane depolarization and Ca²⁺ influx through voltage-dependent calcium channels (VDCC). This calcium influx stimulates the exocytosis of insulin which depends on the target-SNARE proteins Syntaxin 1/4 and SNAP-25 on the plasma membrane and the vesicle-SNARE protein VAMP2 on the insulin granule membrane. These three SNARE proteins form a heterotrimeric complex that facilitates membrane fusion. Glucose metabolism also signals through a metabolic amplifying pathway that increases the amount of insulin secreted in response to the triggering pathway, but without any further changes to Ca²⁺ influx. Diazoxide holds the K_{ATP} channel open, preventing its closure in response to ATP/ADP ratio. **B)** Treatment with diazoxide and KCl elicits the triggering pathway depicted in (A) causing increased cytosolic Ca²⁺ ([Ca²⁺]_c) and insulin secretion. In the further presence of glucose, the amplifying pathway further enhances insulin secretion without increasing [Ca²⁺]_c. Graphs shown are merely an illustrative example based on results from Henquin, et al. (2).

While it is well accepted that triggering is the result of calcium influx, the exact mechanism behind the amplifying pathway is unclear, although it is apparently dependent on glucose metabolism (47). It has been shown that all metabolized nutrients can activate the amplifying pathway, but the second messenger downstream of glucose has remained elusive (2). The search for the mediator(s) of the amplifying pathway has been the focus of much research and certain molecules have evidence both for and against a role, such as inositol phosphates, protein kinase C (51), the ATP/ADP (52, 53) and GTP/GDP (54) ratios, and glutamate (55). Candidates with overall supporting evidence for a role in metabolic amplification include protein acylation (via acyl-CoA) (56-58) and mitochondrial citrate production (59). Reduction of mitochondrial cytochrome C also increases in response to metabolism and increased $[Ca^{2+}]_c$ (60). Finally, NADPH has the potential to be a metabolic coupler (61), although NADPH alone cannot account for the amplifying pathway as NADPH requires the presence of cAMP and ATP to have its effects. Although it is clear that mitochondrial and metabolic signals in the beta cell play an important and complex role in the amplifying pathway [reviewed in (62)], much investigation still remains to fully understand this process.

Cytoskeletal dynamics have recently begun to be investigated for roles in glucose-induced amplification of insulin secretion. The diazoxide paradigm was used in islets in conjunction with pharmacological disruption of F-actin using latrunculin or stabilization of F-actin using jasplakinolide (34). It may seem contradictory, but either polymerization or depolymerization of F-actin using these drugs elicited potentiation of biphasic insulin secretion, consistent with much of the published literature (34). This study highlights how F-actin remodeling may fine-tune and regulate the amount of insulin that is secreted in response the triggering and amplifying mechanisms. Since stabilization of F-actin would be expected to prevent secretion, one explanation for the opposite result may be due to the drug jasplakinolide. Jasplakinolide stabilizes F-actin *in vitro*, but *in vivo* it can also disrupt F-actin and nucleate actin polymerization (63), which may account for its potentiating effects. Two other caveats to the use

of such reagents complicates determination of the role of F-actin in beta cells: 1) the need to pre-treat for a minimum of 20 min, which, in the case of latrunculin, shifts granule distribution to the plasma membrane prior to stimulation (64), and 2) the induction of global changes to F-actin by altering the F/G-actin ratio, as opposed to the localized alterations that occur in response to glucose in beta cells (33, 65). As such, it is important to study beta cells using reagents that act specifically upon key regulators of F-actin dynamics as opposed to the entire F-actin structure in order to elucidate more nuanced regulatory roles of localized cytoskeletal dynamics.

1.3 SMALL RHO FAMILY GTPASES AND REGULATION OF INSULIN SECRETION

Small GTPases (guanosine nucleotide triphosphatases) play many roles in cell biology including, but not limited to cytoskeletal reorganization, vesicle trafficking, and cell growth (66). These GTPases are known as the Ras superfamily due to their sequence homology. The best studied members of this family include Ras, Rho, Rab, Arf, Rap and Ran (67). Specifically of importance to F-actin remodeling is the Rho family, which contains Rho, Rac, and Cdc42. Small GTPases become activated when bound to GTP and remain active until the GTP is hydrolyzed to yield GDP and inorganic phosphate (66). This cycle of GTP binding and hydrolysis is regulated by guanine nucleotide exchange factors (GEFs) which promote the dissociation of GDP and binding of GTP, GTPase activating proteins (GAPs) which stimulate GTP hydrolysis, and GDP dissociation inhibitors (GDIs) which prevent GDP dissociation (68). Once in the activated GTP-bound state, small GTPases will bind to effector molecules to propagate their signal. In pancreatic beta cells, Cdc42 leads to Rac1 activation through p21-activated kinase (PAK1) (43). The third member of the Rho family, RhoA, was initially suggested against having a role in insulin secretion (69), although more recently RhoA was implicated as a negative regulator of insulin secretion (70). The following subsections will describe the Cdc42-PAK1-Rac1 pathway

as it is known to function in beta cells, followed by sections with emphasis on PAK1 and its substrates, Rac1, and finally a discussion of other GTPases with relevance to the beta cell.

1.3.1 The Cdc42-PAK1-Rac1 Signaling Pathway

Using beta cell specific Cdc42 knockout mice, Cdc42 was found to be required for cell polarization responsible for microlumen formation, which is needed for final pancreatic architecture (71). As such, studies focusing upon the role of Cdc42 signaling as it pertains to GSIS in pancreatic beta cells have instead used siRNA-mediated Cdc42 depletion, a more acute method of depletion which avoids developmental artifacts, to determine that Cdc42 in both human and rodent islets is required for specifically for the second phase of insulin secretion (43, 45). More recently, a screen for small molecule inhibitors specific for Cdc42 yielded the compound ML-141 (72). ML-141 is a selective reversibly non-competitive inhibitor of Cdc42. While the ML-141 inhibitor has shown the ability to prevent EGF-stimulated Cdc42 activation in fibroblast cells, the effects in beta cells have yet to be investigated (72).

Cdc42 and Rac1 activation can be measured by a pull down assay using the Cdc42/Rac1 interactive binding domain of PAK1 which specifically binds to GTP-bound Cdc42 and Rac1 (73). In mouse insulinoma MIN6 and rat insulinoma INS 832/13 beta cell lines, Cdc42 is activated within 3 minutes of glucose stimulation and activation declines to basal levels within the following 2 minutes (43, 65, 74, 75). This activation coincides with post-translational modifications like O-glycosylation, carboxymethylation and prenylation (65, 76). Depletion of Cdc42 from either mouse or human isolated pancreatic islets causes a selective loss of second-phase insulin secretion (43, 45). The actual signaling factor that causes Cdc42 activation specifically in response to glucose and how this controls second-phase is not yet clear, although it is known that the signal requires glucose metabolism since non-metabolizable glucose analogues or KCl-depolarization induced Ca^{2+} influx fail to induce Cdc42 activation (43). Recent findings suggest a role for the GEF Cool-1/ β Pix in mediating Cdc42 activation (74). Within 2 min of

glucose stimulation, the association between Cdc42 and β Pix increases by ~3-fold compared to unstimulated conditions (74). This binding event immediately precedes Cdc42 activation at 3 min, consistent with a role for β Pix as a Cdc42 GEF. siRNA-mediated depletion of β Pix ablates glucose-induced Cdc42 activation and decreases glucose-stimulated insulin secretion (74), although the upstream activator of β Pix is unknown. Activated Cdc42 directly binds to PAK1, which then becomes activated through autophosphorylation on Thr 423 after 5-10 min of glucose stimulation (43, 73). Islets from PAK1 knockout mice exhibit drastically reduced second-phase GSIS, placing PAK1 downstream of Cdc42 in the regulation of second-phase insulin secretion (43, 45). PAK1 activation is required for Rac1 activation, and Rac1 is activated after 15-20 min of glucose stimulation (43).

Under unstimulated conditions, both Cdc42 (44, 65, 77) and Rac1 (78) are maintained at a low level of activation via sequestration by GDIs. Two GDIs have been identified in beta cells that regulate Cdc42: Caveolin-1 (77) and RhoGDI (44). The subcellular distribution of Cdc42 impacts its activation since its regulatory GDIs are also differentially localized to cytosol and plasma membrane. Under unstimulated conditions the majority of Cdc42 resides in the cytosol, and the remainder is located at the plasma membrane and secretory granule membrane (74, 77). Inactive Rac1 is localized mainly in the cytosol (79). Caveolin-1 is a membrane bound protein important for forming caveolae in many cell types, but also has a major role in signal transduction (80-82). Caveolin-1 has been found to function as a GDI for Cdc42 in several cell types, including islet beta cells (77, 83). Evidence for this GDI role for Caveolin-1 in beta cells stems from studies showing that Caveolin-1 associates specifically with Cdc42 on the insulin granule membrane (77). When Caveolin-1 levels are depleted in islet beta cells, Cdc42 is aberrantly activated and basal insulin secretion inappropriately increases by 2-fold (77). The other identified GDI, RhoGDI, is cytosolic and acts as a GDI for Cdc42 (44) and Rac1(79) in beta cells.

RhoGDI was found to interact with the cytosolic pools of Cdc42 and Rac1 in MIN6 beta cells, but regulates them differently depending on the duration of glucose stimulation (44, 79).

For example, RhoGDI becomes phosphorylated on tyrosine 156 within 3 min of glucose stimulation to dissociate from Cdc42 but not Rac1 (44). After 10-20 min of glucose stimulation, RhoGDI becomes phosphorylated on serines 101 and 174 and dissociates from Rac1 (44). In bovine brain, the serine phosphorylation sites on RhoGDI were identified as PAK1 target sites, which fit well in the model where PAK1 is required for Rac1-RhoGDI dissociation (44, 84). Furthermore, the dissociation of RhoGDI from Rac1 occurs only in response to the amplifying pathway, but not the triggering pathway, supportive of a glucose-dependent regulation of RhoGDI-Rac1 complexes (44). Mutation of tyrosine 156 to phenylalanine blocked glucose-induced Cdc42 dissociation and similarly, mutation of serines 101 and 174 to alanine blocked Rac1 dissociation (44). It is likely that serines 101 and 174 are phosphorylated on RhoGDI in response to the amplifying pathway, thus conferring glucose-specific Rac1 dissociation, although this has yet to be tested directly (44). Nevertheless, MIN6 or INS-1 cells, and mouse islets depleted of RhoGDI all showed a robust potentiation of GSIS, specifically in the second phase in islets, consistent with a selective role for RhoGDI in the amplifying pathway (44, 79). The enhanced secretion was correlated with potentiated Cdc42 activation, which presumably enhances PAK1 and Rac1 activation, although these events have not yet been investigated under RhoGDI depletion in beta cells (44).

Currently, there are few characterized downstream targets of Cdc42 in the beta cell. In addition to PAK1 activation, Cdc42 may activate N-WASP and the Arp2/3 complex in beta cells to cause glucose-stimulated insulin secretion and actin remodeling (85). Cdc42 also binds to the v-SNARE VAMP2 on the insulin granule via a direct interaction, regardless of GTP- or GDP-bound status (86). After glucose stimulation, Cdc42 translocates to the plasma membrane where it is activated and may have a role in targeting granules to the plasma membrane for secretion, since Cdc42, VAMP2 and Syntaxin 1A can all participate in a complex (86). The reason(s) that Cdc42 activation is required specifically for second-phase glucose-stimulated insulin secretion is not completely clear. Cdc42 is likely activated early (3 min after stimulation) in order to begin

signaling cascades, one of which is through PAK1, that either mobilize granules toward the plasma membrane for release, enhance the release competence of granules that are nearby the plasma membrane, or both.

1.3.2 The Role of PAK1 Signaling in the Beta Cell

PAK1 belongs to a family of PAK isoforms that are grouped into two main categories based upon sequence homology; Group I PAKs include PAK1, PAK2, and PAK3, while Group II includes PAK4, PAK5, and PAK6. PAK1 is the most studied family member. It is important to point out that the 21 kDa proteins that activate PAK1 are the small Rho GTPases Rac and Cdc42, not the cyclin-dependent kinase inhibitor p21. Depending on the cell type, either Cdc42 or Rac1 are able to interact with the Cdc42/Rac1 interactive binding domain of PAK1, which in turn induces a conformational change where the regulatory domain of PAK1 dissociates from the kinase domain, triggering PAK1 activation (73). In beta cells, Cdc42 activates PAK1, and PAK1 is necessary for Rac1 activation (43). Recently, PAK1 levels were observed to be decreased in islets from type 2 diabetic humans, and human islets treated with the PAK1 inhibitor IPA3 showed attenuated second phase insulin release (45). This study solidified the importance of PAK1 in human type 2 diabetes, specifically in pancreatic islet beta cells. Moreover, mouse islets lacking PAK1 (from PAK1 knockout mice) also exhibited reduced second-phase glucose-stimulated insulin secretion (45). Islets from PAK1 knockout mice showed defective glucose-induced ERK activation in beta cells (45). PAK1 could be regulating ERK activation through multiple mechanisms including PAK1 activation of Raf and/or MEK and Raf/MEK/ERK binding to PAK1 as a signaling scaffold (87, 88). In addition to signaling through ERK, PAK1 in other cell types has been demonstrated to utilize several other substrates for phosphorylation, including LIM kinase (89), Filamin A (90), myosin light-chain kinase (MLCK) (91), RhoGDI (84), MEK (87, 92), and the p41-Arc subunit of the Arp2/3 complex (93). As such, it remains possible that

the modification of one or more of these targets by PAK1 could contribute to second-phase insulin secretion.

1.3.3 The Role of Rac1 Signaling in the Beta Cell

Rac1 activation occurs within 15-20 min of glucose stimulation and has been shown to be required for glucose-stimulated insulin secretion (69, 78, 94). In the rat beta cell line INS-1, expression of dominant negative Rac1 (T17N-Rac1) blunted glucose- and forskolin plus glucose-induced secretion, but had no effect on KCl-induced secretion, demonstrating the glucose-specificity of Rac1 (78). Subsequently, a study using inhibitors against Raf-1 and farnesyltransferase or using knockdown of ERK showed that these proteins are important for glucose-induced Rac1 activation in the rat beta cell line INS 832/13 (95). Since Rac1 is activated after second phase has already begun, it might be that Rac1 is not required for the initiation of second phase, but may play a role in sustaining secretion. Potential effectors that Rac1 acts through to mediate glucose-stimulated insulin secretion include Gelsolin (96) and the protein complex Nox (97). Rac1-dependent PIP₂ production was suggested to be a driving force to inactivate Gelsolin and sequester it at the PM (98). In this manner, F-actin could be severed, then re-polymerized, and severed again based on the cycling of Rac1 GTP/GDP loading (98). The Nox complex is responsible for superoxide production and the complex assembly depends on Rac1 activation (76). Inhibition of the Nox complex led to decreased glucose-stimulated insulin secretion, therefore Nox is believed to be a positive effector of insulin secretion downstream of Rac1 (97).

1.3.4 Other Small GTPases

Multiple other small GTPases have been investigated for their roles in vesicle trafficking, cytoskeletal dynamics and exocytosis. This section will briefly highlight candidates for regulation of biphasic insulin secretion from islets, including Arf6, RhoA, Rap1, Rab, and RalA.

The small GTPase Arf6 was recently reported to become activated within the first minute of glucose stimulation and was proposed to be an upstream activator of the Cdc42 pathway (75). However, Arf6 was affiliated with both glucose and KCl-stimulated insulin secretion (75), and not solely glucose stimulated as would have been consistent with the responsiveness of Cdc42 (43). While Cdc42 and Rac1 are positive regulators of secretion in beta cells, the role of the other major Rho family GTPase, RhoA, has remained unclear, given that conflicting reports argue its role as both a positive and a negative regulator of secretion in beta cells (70, 99). Rap1 has also been shown to function as a positive regulator of insulin secretion. Rap1 is activated downstream of GLP-1 signaling in beta cells due to cAMP-dependent activation of the Rap1 GEF Epac2 (100, 101). Rap1 is required for the full potentiation of glucose-induced insulin secretion by cAMP (100) and also has a role in beta cell proliferation through the mTOR pathway (102).

Finally, RalA and the Rabs have recently been shown to be important in insulin secretion and may have ties to the Cdc42 pathway and F-actin remodeling, respectively. RalA is a member of the Ras-family GTPases and is thought to have roles in exocytotic processes (103). In mouse islets, depletion of RalA results in reduced first and second phase of insulin secretion, while overexpression of RalA in MIN6 cells enhances insulin secretion (104). The mechanism of RalA action involves its activation by the Ral GEF RalGDS and signaling through Arf6 and phospholipase D1, although the connection of RalA to Arf6 has yet to be investigated in beta cells (105). In the case of the Rabs, although many have roles in membrane trafficking, Rab27a and Rab3a are of particular interest for insulin secretion. Rab3a is important for the size of the readily releasable pool of granules, while Rab27a is important for refilling the releasable pool (106). This could be explained by Rab27a signaling downstream to myosin Va to promote insulin granule transport to the plasma membrane, linking this Rab to the F-actin cytoskeleton (107, 108).

1.4 F-ACTIN AS A REGULATOR OF EXOCYTOSIS

F-actin has been recognized as a regulator of exocytosis for more than 40 years (1, 109-112). Use of F-actin disrupting drugs like cytochalasins, latrunculins, clostridium C2 toxins, and F-actin polymerizing drugs like phallotoxins and jasplakinolide have proven useful in delineating F-actin's role(s) in secretory/exocytotic processes across multiple cell types (1, 65, 112). These cell types include beta cells and acinar cells (113), chromaffin and PC12 cells (114, 115), melanotrophs and lactotrophs (116, 117), neutrophils (98, 99, 118), platelets (119), endothelial cells (120), neurons (121), adipocytes (122), and myocytes (123). Landmark work by Orci, et al. showed electron micrographs of islet beta cells depicting F-actin in the form of a meshwork barrier just beneath the plasma membrane and in the form of tracks on which granules could translocate to the PM (1). Aside from beta cells, most research on F-actin in secretion has been conducted in cultured adrenal chromaffin cells and PC12 cells. In chromaffin cells, F-actin also forms a cortical ring beneath the plasma membrane and colocalizes with cytoskeletal proteins like myosin, caldesmon (109, 124, 125) Gelsolin and Scinderin (126), and fodrin (127).

1.4.1 Positive and Negative Roles of F-actin in Secretion

To sustain insulin release, mature insulin granules in intracellular storage pools must be mobilized toward the plasma membrane. This process coincides with glucose-induced remodeling of the actin cytoskeleton (33, 65, 128). In this sense, F-actin 'remodeling' in beta cells encompasses the simultaneous localized depolymerization and polymerization of F-actin across the cell in a concerted manner, such that the F/G-actin ratio does not change in response to glucose (65). F-actin was long thought to function solely as a barrier to regulate insulin granule accumulation at the plasma membrane (1, 109), such that its depolymerization results in more morphologically docked granules and may confer enhanced release competence to granules (34, 64). Even though drug treatment studies suggest that F-actin is simply a blockade against

secretion, evidence of positive effects of the cytoskeleton in stimulus-induced insulin secretion exists as well (129-132). Glucose has been shown to increase F-actin in some cases and decrease F-actin in others (130, 131, 133).

1.4.2 F-actin, Beta Cell-Cell Contacts, and the Regulation of Basal Insulin Secretion

Control of basal insulin secretion, the amount of insulin secreted under non-stimulatory or sub-threshold conditions, is a critical part of physiology (134). Dysregulation of basal insulin secretion occurs in type 2 diabetes (134) and also in certain genetic disorders where the K_{ATP} channel is mutated (135). Aberrantly increased insulin secretion under normal blood glucose conditions can cause hypoglycemia acutely, but over time may contribute to insulin resistance in the peripheral tissues (134). The cell's ability to keep basal insulin secretion at a normal low level seems to be linked to the F-actin cytoskeleton and cell-cell communication.

Recently, the actin cytoskeleton has been linked to roles in basal exocytosis through control by several factors: β Pix (74), Cav-1 (77), focal adhesion kinase (FAK) (136) and EphA-Ephrin-A signaling (132). Depletion from beta cells of either β Pix or Cav-1, which are both regulators of Cdc42 activation, leads to dysregulated basal insulin secretion (74, 77). Pharmacological inhibition of FAK caused an increase in basal insulin secretion which was related to disrupted beta cell F-actin structure due to discontinuous cell-cell contact (136, 137). EphA5-Ephrin-A5 signaling mediates a paracrine signaling pathway that regulates insulin secretion. Decreased cell-cell contact in beta cells decreases EphA5 phosphorylation levels, which leads to increased basal secretion (132). The EphA5-Ephrin-A5 signaling mechanism seems to be required for normal beta cell function as knockout of Ephrin-A5 in mice largely inhibited glucose-stimulated insulin secretion and caused whole-body glucose intolerance (132). Other forms of cell-cell contact, such as gap junctions formed by connexin 36, have been implicated in basal insulin secretion (138-141). In islets isolated from connexin 36 knockout mice, basal insulin secretion was increased (147). In another study, under high-fat diet

conditions, mice lose protein levels of connexin 36, leading to increased basal secretion, suggesting this may be a potential effect in human obesity and type 2 diabetes disease progression (138). Taking these findings together, there is a crucial link between F-actin cytoskeletal regulation, cell-cell contact, and control of basal and stimulated insulin secretion.

A body of work suggests that maintenance of Cdc42 in its inactive state is important to maintain the normally low basal insulin secretion. For example, siRNA-mediated knockdown of the Cdc42 GEF β Pix in beta cells reduced both basal and glucose-stimulated insulin secretion and prevented glucose-stimulated Cdc42 activation (74). Furthermore, knockdown of the GDI Caveolin-1 in both MIN6 cells and mouse islets caused a significant increase in basal insulin secretion, demonstrating that control of Cdc42 activation is important for keeping basal secretion in check (77). It may be germane to point out that while Cdc42 has roles in both basal insulin secretion and second-phase GSIS, it is not clear whether these two functions lie within the same signaling pathway or are distinct from one another.

1.5 ACTIN-BINDING PROTEINS IN EXOCYTOSIS AND GRANULE TRAFFICKING

Glucose elicits F-actin remodeling in beta cells, while stimulation using KCl does not (65). Consistent with this, Cdc42 is activated by glucose to signal to downstream targets PAK1 and Rac1, leading to second-phase insulin secretion (43, 44). Since F-actin may also be necessary to inhibit insulin secretion under low glucose conditions (130), a dynamic role for F-actin in corralling insulin granules via glucose-stimulated remodeling has been proposed (35). In this sense, the role of F-actin may be as a barrier under unstimulated conditions, but become a facilitator of insulin secretion during glucose stimulation. Since insulin granules are known to interact with F-actin filaments (142), this could be one way granules are mobilized to the plasma membrane in a regulated manner. As previously discussed, F-actin remodeling is known to couple granule mobilization to the SNARE exocytosis machinery (33, 64, 86, 143). Therefore, it

is pertinent to analyze proteins that control the polymerization and/or depolymerization of F-actin structures and where and when these events occur. The idea that F-actin severing and stabilizing proteins may underlie glucose-stimulated F-actin remodeling fits with the current model and also leaves room for the potential modifiers of F-actin dynamics in the beta cell.

1.5.1 F-actin Severing Proteins

Glucose-induced F-actin remodeling inherently must involve regulated depolymerization of F-actin. The best studied F-actin severing proteins that may play a role in this process include Cofilin, Gelsolin, and Scinderin. Cofilin's F-actin severing activity is regulated through phosphorylation on Ser 3 by LIM kinase which inhibits Cofilin, and dephosphorylation by the phosphatase Slingshot which activates Cofilin (144). Cofilin is usually thought to be regulated upstream by PAK1 activation of LIM kinase, but recent findings suggest that in the beta cell PAK1 is dispensable for the phosphorylation state of Cofilin (45). This is in contrast to skeletal muscle where PAK1 was required for the dephosphorylation of Cofilin phosphorylation (45). Whether Cofilin or its phosphorylation plays any role in GSIS remains to be investigated.

Gelsolin is the founder of a family of calcium-activated actin-severing and capping proteins, first cloned in 1979 (145, 146). This protein family includes Gelsolin, Scinderin, CapG, and many others (146), although only Gelsolin and Scinderin are known to be expressed in beta cells. Gelsolin consists of 6 globular domains (S1-6), each of which binds calcium; S2 binds phosphoinositides (146). Since its discovery, Gelsolin has been implicated in different aspects of cell biology including roles in cancer, binding to androgen receptor, and F-actin remodeling (147). There are two isoforms of Gelsolin encoded as different mRNAs from the same gene: a cytosolic isoform and a secreted plasma isoform which contains a 50 residue N-terminal extension (148, 149). Plasma Gelsolin in the blood is thought to be important for reducing inflammation after tissue injury and in recovery from certain infections (150), although the mechanisms of these functions are not well understood. The larger portion of Gelsolin literature

relates to its cytosolic form and its role in F-actin remodeling. Gelsolin is ubiquitously expressed and has been implicated in insulin secretion from beta cells (96, 151), but the affected phases of secretion and its mechanistic role in insulin secretion remains largely unstudied (96, 152-154).

Scinderin shares nearly 60% sequence identity to Gelsolin (155). Unlike Gelsolin, Scinderin expression is largely restricted to neuroendocrine, intestinal, and kidney tissue (154, 156). Despite their structural similarity, Gelsolin and Scinderin are differentially regulated by calcium and Scinderin plays a larger role in secretion from chromaffin cells (126, 157, 158). Scinderin becomes activated at lower levels of calcium ($0.5\mu\text{M Ca}^{2+}$) than Gelsolin ($\sim 1\text{-}3\mu\text{M Ca}^{2+}$) (158). In patch-clamped primary beta cells, peptides derived from Scinderin's PIP_2 binding domain were shown to inhibit calcium- or $\text{GTP}\gamma\text{S}$ -induced insulin secretion, potentially implicating Scinderin in glucose-stimulated insulin secretion (159).

1.5.2 F-actin Stabilizing Proteins

The two major F-actin stabilizing proteins which may have potential roles in beta cell F-actin remodeling are α -Fodrin and Filamin A. α -Fodrin, also known as spectrin, cross-links F-actin under low calcium and releases actin upon calcium influx in chromaffin cells (127). α -Fodrin also binds to Syntaxin 4 *in vitro* and in rat adipocytes (160, 161), which implicates it in the modulation of SNARE complex formation and may link this complexation to F-actin. α -Fodrin can be cleaved by calpain and increased cleaved α -Fodrin is found in islet beta cells from type 2 diabetic donors, suggesting compromised cellular cytoskeletal structure (162). Filamin A is the most potent F-actin cross-linking protein identified and interacts with Cdc42, PAK1 and Rac1, all of which are vital for second-phase insulin secretion (43, 163-165). PAK1 is known to phosphorylate Filamin A *in vivo* and *in vitro*, regulating its F-actin cross-linking activity (90), although this has yet to be shown in beta cells.

1.5.3 F-actin Associated Proteins

In addition to F-actin severing and stabilizing proteins, there is a set of proteins that have the potential to play critical roles in insulin secretion that should be discussed; these include Arp2/3, Myosin light-chain kinase (MLCK) and Myosin Light-Chain 2 (MLC2), and FAK. The Arp2/3 complex has direct links to Cdc42 signaling in other cell types and could therefore be involved in glucose-induced F-actin remodeling. Arp2/3 has been implicated in secretion from neuroendocrine cells (114), and recently was suggested to play a critical role in glucose-stimulated insulin secretion, potentially in second phase through interaction with Cdc42 (85).

MLCK and MLC2 are implicated in glucose-stimulated insulin secretion and hold the potential for effects on F-actin remodeling and granule mobilization. Myosin IIA is the most studied non-muscle myosin and is involved in F-actin remodeling and exocytosis (166, 167). Myosin IIA is comprised of two identical heavy chains coiled together by a long tail domain each with a myosin head domain, two essential light chains, and two regulatory light chains (Figure 1-4A). The regulatory light chain is referred to as myosin light chain 2 (MLC2) and is phosphorylated by MLCK (Figure 1-4B); an event implicated in insulin secretion (168). In support of this, an increase in MLC2 phosphorylation on Ser 19 was observed in beta cells (169). Phosphorylation of MLC2 Ser 19 increases the ATPase activity of Myosin IIA (170). Ca^{2+} -calmodulin directly activates MLCK, and ERK has been shown to phosphorylate and increase the sensitivity of MLCK to Ca^{2+} -calmodulin (171). MLCK activity is increased by glucose in beta cells, suggesting a potential link between ERK and MLCK in glucose-stimulated insulin secretion (168). PAK1 has also been shown to phosphorylate MLCK, but has an inhibitory effect on MLCK (91). In addition to Myosin IIA, Myosin Va has been investigated in beta cells (128). Myosin Va was shown to be a positive regulator of granule localization to the plasma membrane and subsequent insulin secretion (128).

Finally, FAK regulation of focal adhesion remodeling has implications for indirect regulation of F-actin remodeling and insulin secretion. In MIN6 beta cells, FAK inhibition was

shown to prevent glucose-stimulated F-actin remodeling (137) and led to increased basal secretion and decreased GSIS (136). Recently, islets of beta cell-specific FAK knockout mice were found to exhibit decreased ERK activation, reduced number of docked insulin granules that correlated with deficient insulin secretion, and impaired glucose induced cortical F-actin reorganization (172). Thus, FAK likely acts in coordination with F-actin remodeling in order to regulate glucose-stimulated insulin secretion, but its linkage to Cdc42 and PAK1 remains unknown.

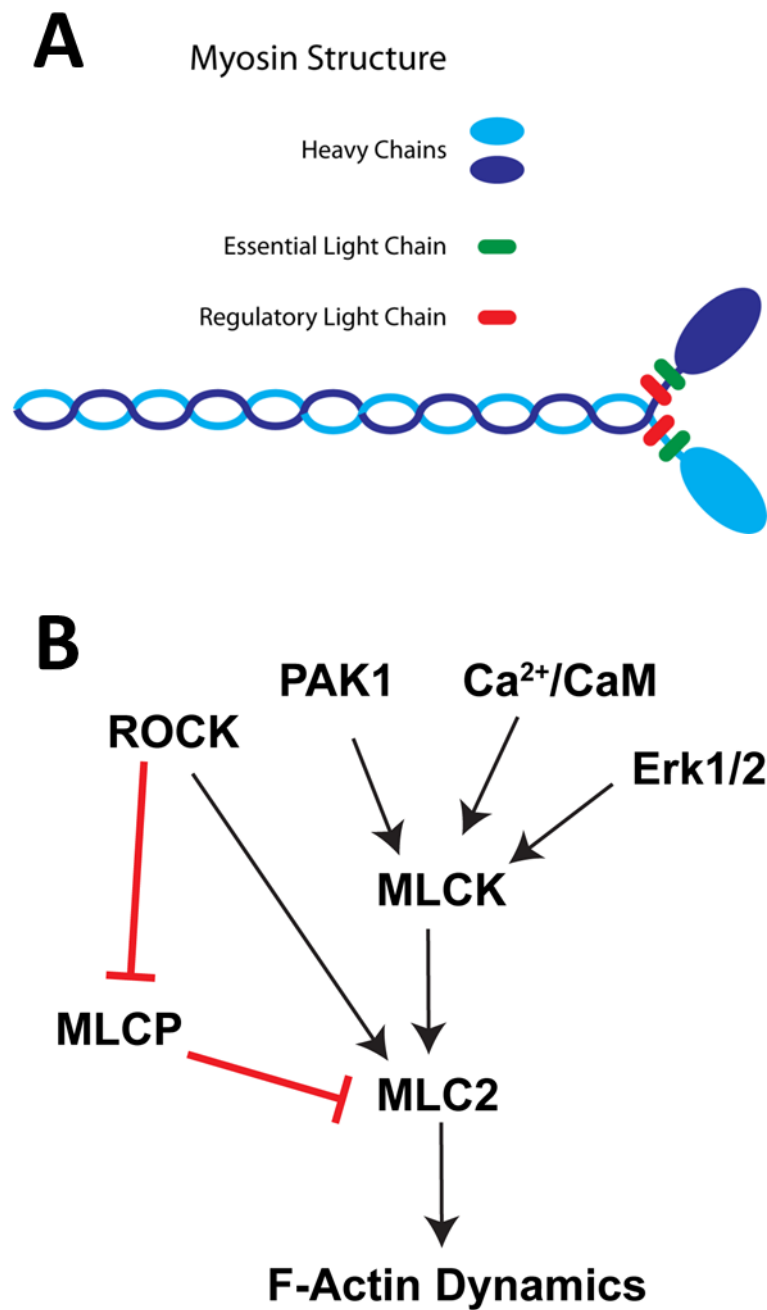


Figure 1-4. Structure of myosin IIA and signaling. A) Depiction of the structure of Myosin IIA. B) Schematic of myosin light chain 2 (MLC2) regulation by different signaling pathways.

1.6 ROLE OF ERK IN ACTIN REMODELING AND SECRETION

1.6.1 The Ras-Raf-MEK-ERK Pathway in the Beta Cell

Extracellular signal-regulated kinase (ERK) is a member of the mitogen-activated protein kinase (MAPK) family that functions in cell growth and survival, embryogenesis, and differentiation across many cell types (173). The pathway leading to ERK activation in the beta cell involves various signals including Ca^{2+} influx and cAMP production. In the beta cell, cAMP leads to activation of the small GTPase Rap1a which can activate B-Raf (174). Raf then phosphorylates the MAP/ERK kinase (MEK) which subsequently phosphorylates ERK (Figure 1-5) (173). PAK1 can feed into this pathway as well by phosphorylating both Raf-1 and MEK1 to promote activation of each protein (87, 175, 176). The involvement of Rap1a implicated the cAMP-responsive GEF Epac2 in B-Raf signaling and this pathway has recently been demonstrated in GLP-1 stimulated human islets (177).

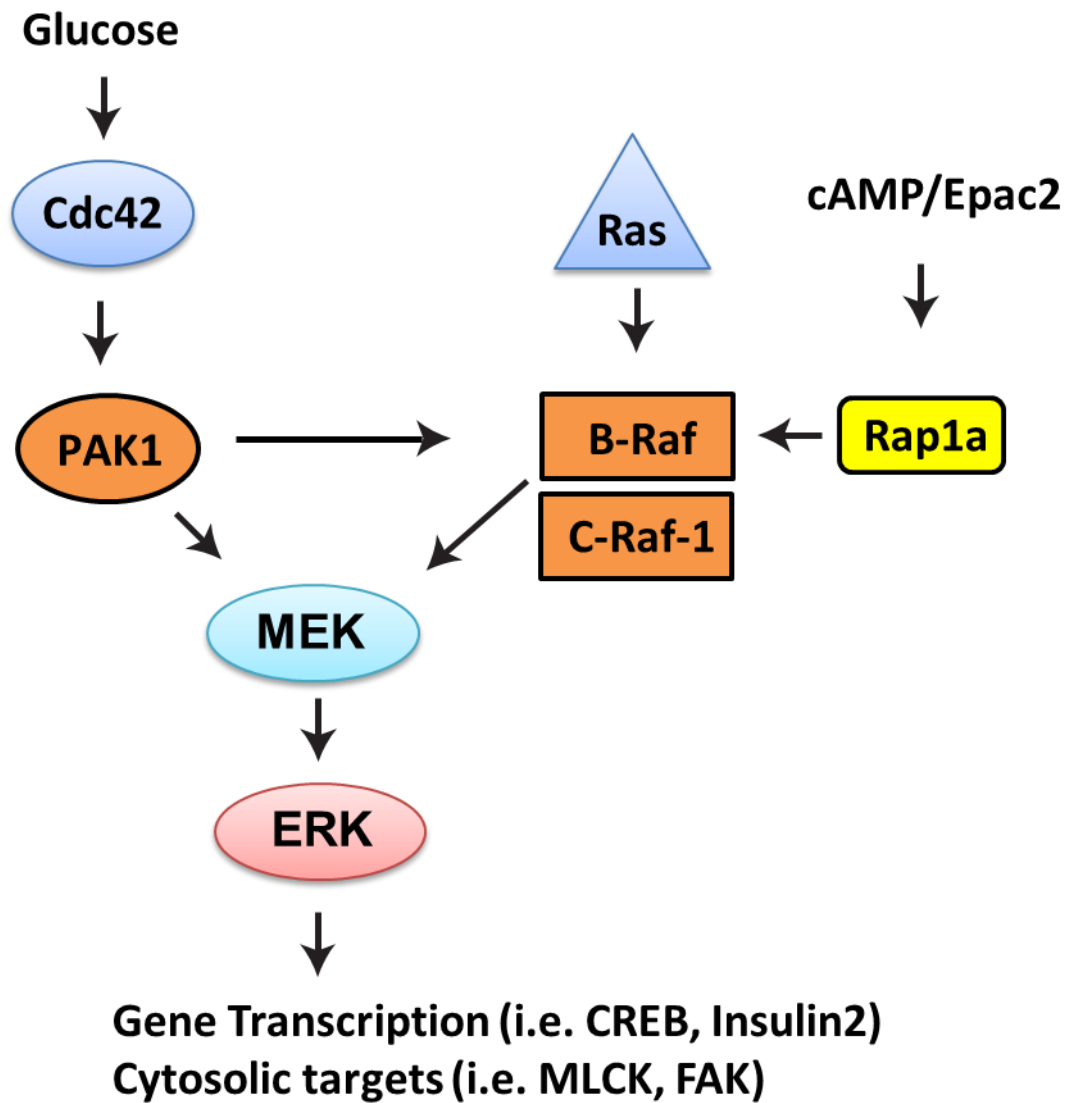


Figure 1-5. The Cdc42-PAK1 and Ras-Raf pathways feed into MEK-ERK signaling. In the beta cell, glucose activates Cdc42 by 3 min of stimulation which then leads to PAK1 activation by 5 min. Raf is activated by upstream signals in many cell types, while in beta cells glucose is thought to mainly activate B-Raf. Active PAK1 can phosphorylate Raf and MEK to promote Raf-MEK signaling which culminates in ERK activation. Active ERK can then target downstream gene transcription events and cytosolic targets.

More than 15 years ago, ERK was shown to be activated in response to glucose in beta cells (178, 179). However, initial studies with first-generation inhibitors yielded data that suggested ERK activation was not necessary for acute insulin secretion (178, 179). These initial studies concluded that active ERK translocated to the nucleus to exert effects upon transcription, although did also acknowledge that a larger proportion remained in the cytoplasm (178-180). More recent studies using lower concentrations of PD98059 (20 μ M) in rat islets revealed a necessity for ERK in glucose-stimulated insulin secretion (180); RNAi-targeting of ERK resulted also in attenuated GSIS and Rac1 activation (95). Recently, a new selective MEK inhibitor has been generated, PD0325901, that will be useful in reconciling the role of ERK in GSIS (181).

As a result of the controversy surrounding a role for ERK activation in GSIS, the effects of ERK activation in pancreatic beta cells on F-actin dynamics/signaling and granule translocation to SNARE sites have been relatively understudied. In the case of ERK inhibition, use of high 100 μ M doses of PD98059 were concluded to have no impact on glucose-induced F-actin remodeling, although only stress fibers were analyzed, leaving the possibility of effects on cortical actin in the midplane of the cell (96). Although it is clear that ERK has some connection to F-actin, since activate ERK localizes to the tips of F-actin processes in beta cells (96, 136). Depolymerization of F-actin using latrunculin seems to potentiate glucose-induced ERK activation, while stabilization of F-actin with jasplakinolide attenuates ERK activation, although the reason for this is unclear (96, 137). It is interesting to note that both of these drugs can potentiate glucose-stimulated insulin secretion in beta cells, although it is unknown how ERK activation impacts secretion under either condition (34, 64, 65).

1.6.2 ERK Targets Actin Regulatory Proteins

ERK has been shown to phosphorylate synapsin I, cortactin, MLCK, FAK, and paxillin. Although synapsin I and II have now been suggested against having much role in glucose-stimulated insulin secretion (182), the other F-actin-related ERK targets such as FAK and MLCK

have roles (168, 172); cortactin has yet to be investigated in beta cells (183, 184). ERK can also phosphorylate FAK and this disrupts FAK-paxillin binding (185-187). Since FAK is required in beta cells for cortical F-actin dynamics, basal and stimulated insulin secretion, and focal adhesion remodeling (136, 137, 172), there exists potential for input from the ERK pathway through FAK signaling. Additionally, MEK associates with paxillin and facilitates Raf-MEK-ERK signaling (188), and depletion of paxillin reduced glucose-stimulated insulin secretion and ERK activation in primary rat beta cells (136).

1.7 SNARE-MEDIATED INSULIN EXOCYTOSIS

Insulin exocytosis is mediated by a group of proteins termed soluble N-ethylmaleimide sensitive factor attachment receptor (SNARE) proteins. The concept SNARE proteins in regulated exocytosis was first elucidated in 1993 and rapidly confirmed by multiple groups and in many diverse cell types, including beta cells (189-194). This mechanism is well-conserved from yeast to humans where SNARE proteins are required to facilitate the fusion of lipid bilayer membranes (192). There are two main groups of SNARE proteins, target membrane t-SNAREs and vesicle-associated v-SNAREs. In beta cells the t-SNAREs Syntaxin and synaptosomal-associated protein of 25 kDa (SNAP-25, as well as its homolog SNAP-23) are localized to the plasma membrane whereas the v-SNARE VAMP2 (also known as synaptobrevin 2) is localized to the secretory vesicle (Figure 1-6). Syntaxins have three N-terminal alpha helices denoted Ha, Hb, and Hc (Habc) followed by a linker domain and then the H3 SNARE helix domain that associates with the other SNARE proteins (192). VAMP2 is a relatively small protein (~18 kDa) which mainly contains a single alpha-helical SNARE domain. VAMP2 and Syntaxin each donate their one alpha-helix and SNAP-23/25 contributes two alpha-helices to form a coiled four helical heterotrimeric complex that brings the plasma membrane and vesicle membranes in close enough proximity for fusion (195). The so-called 'priming' step involved in the SNARE complex

formation is ATP-dependent and the final initiation of regulated exocytosis requires calcium influx (21), although the exact mechanisms by which this calcium influx causes membrane fusion are not fully understood. In addition, other priming factors are important for SNARE-mediated membrane fusion such as the Munc13 proteins and Doc2 β , which also have calcium-binding activity that could aid in sensing increased cytosolic [Ca²⁺] (196).

1.7.1 SNARE Requirement in Biphasic Insulin Secretion

In beta cells, the necessary SNARE isoforms are Syntaxin 1 and 4, SNAP-25 (and SNAP-23), and vesicle-associated membrane protein 2 (VAMP2). Indeed, the levels of multiple SNARE proteins are reduced in islets from type 2 diabetic donors for example Syntaxins 1 and 4, SNAP-25, and VAMP2, highlighting the functional importance of these proteins in the disease state (197). In general, the Syntaxin 1 is required for first-phase insulin secretion only, while Syntaxin 4 is necessary for both phases of insulin secretion (39, 42). The Syntaxins are regulated by a group of nSec1/Munc18 (SM) proteins. The differential requirement of Syntaxin 1 versus Syntaxin 4 in biphasic secretion could be the result of SM protein regulation. Syntaxin 1 and Syntaxin 4 are regulated by Munc18-1 and Munc18c, respectively, and these complexes are mutually exclusive (196, 198, 199). Munc18-1 was recently discovered to be required for first-phase insulin secretion, which is consistent with its role as a regulator of Syntaxin 1 (198). Munc18c is required specifically for second-phase insulin secretion (200), which may underlie Syntaxin 4's role in second phase, but the mechanism of Syntaxin 4's role in first-phase remains an open question. It is hypothesized that upon stimulation, post-translational modification of SM proteins causes dissociation (in the case of Munc18c) or a binding mode shift (in the case of Munc18-1) to assist in the opening and binding of Syntaxin to its cognate v-SNARE VAMP2 (Figure 1-6) (196). Post-translational modification of the Syntaxin proteins also contributes to their activity, as glucose-induced production of nitric oxide causes S-nitrosylation of Syntaxin 4 in beta cells, increasing Syntaxin 4 accessibility to VAMP2. Another layer of regulation comes

from the protein Doc2 β , which regulates Munc18-1 and Munc18c (196, 199, 201, 202). Genetic ablation of Doc2 β results in attenuated first- and second-phase insulin secretion (199). Currently, the mechanism for how Doc2 β regulates both phases is under investigation but likely has to do with its regulation of the Munc18 and the Munc13 proteins. Recently, Munc13-4, part of the Munc13 family of priming factors, was shown to interact with the H3 SNARE domains of Syntaxins 1 and 4 in a calcium-dependent manner (203). Since Munc13-4 interacts with Rab27a, a key protein regulating vesicle trafficking, this could underlie a mechanism linking GTPases to SNARE-mediated exocytosis (204).

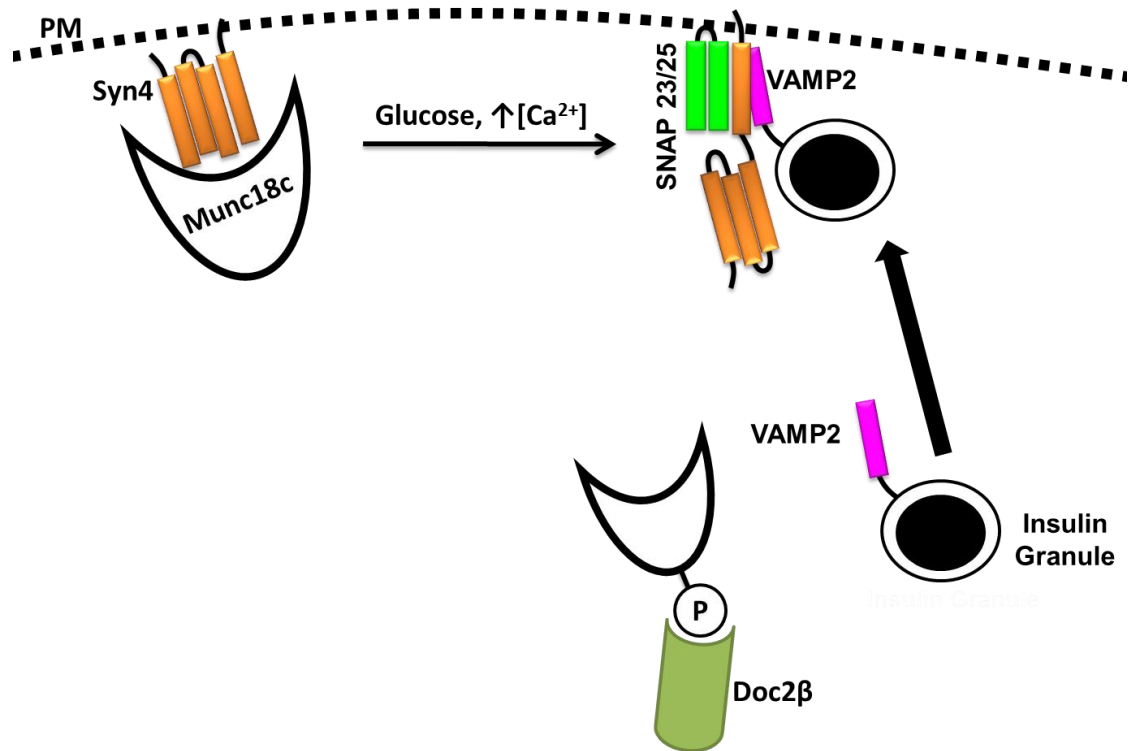


Figure 1-6. Regulation and mechanism of SNARE complex formation. The SNARE complex forms first from the binding of Syntaxin 4 (Syn4) and SNAP-23/25 after stimulus induced release of Syn4 from Munc18c. Phosphorylated Munc18c is bound by Doc2B. VAMP2 can then bind to Syn4-SNAP-23/25 complexes to form the SNARE complex and elicit membrane fusion.

1.7.2 Linkage of F-actin Remodeling to SNARE-mediated Secretion

The SNARE proteins involved in insulin secretion are linked to F-actin by direct and indirect interactions. These SNARE-F-actin interactions are thought to regulate SNARE protein function and influence insulin secretion (35). Immunoprecipitation of Syntaxin 1, Syntaxin 4, and SNAP-25 from beta cells show co-precipitation of actin (33, 64, 137, 143, 205). Of interest is the dynamic nature of the F-actin-SNARE interaction after glucose stimulation. After 5-10 minutes of glucose stimulation, F-actin dissociates from the Syntaxins and SNAP-25, but reassociates within 30 minutes after stimulation (33, 64, 137). In the case of Syntaxin 4 this binding has been shown to be direct, while Syntaxin 1 seems to interact through an indirect mechanism (33, 64, 65). Direct binding of F-actin to Syntaxin 4 was determined to occur at the N-terminus of Syntaxin 4, likely through the Hb helix while direct binding of F-actin to VAMP2 was not detected *in vitro* (64). The Syntaxin 4-F-actin interaction seems to be selective for Syntaxin 4, since all other SNAREs tested so far (Syntaxin 2 and 3, SNAP23, VAMP8) have not bound F-actin directly *in vitro* (119, 205). In beta cells, evidence for Syntaxin 4 binding to F-actin and not G-actin comes from treatment with the actin depolymerizing drug latrunculin, which abrogates the co-precipitation of actin with Syntaxin 4, showing that Syntaxin 4 will not bind to monomeric G-actin (64, 143). Artificial disruption of Syntaxin 4-F-actin complexes in beta cells using a GFP-tagged truncation of Syntaxin 4 corresponding to the F-actin binding (Syntaxin 4 residues 39-112) site led to increased secretion and increased VAMP-bound insulin granule accumulation at the cell surface, suggesting that F-actin binding to Syntaxin 4 played a role in limiting granule access to the PM (64). Syntaxin 4 and 1 can also form heteromeric complexes with Cdc42 and VAMP2 *in vitro*, presenting a potential avenue for localized coupling SNARE proteins with Cdc42-mediated F-actin remodeling (86, 206).

1.8 RATIONALE AND CENTRAL HYPOTHESIS

The *rationale* for the studies in this dissertation is that further understanding of the role for F-actin in regulating insulin secretion will not only help close a gap in understanding in the field of beta cell biology, but also uncover potential new therapeutic targets for diabetes treatment strategies. The central hypothesis of this dissertation was formulated based on my preliminary data and the published literature described above regarding the known requirement for small Rho GTPases, SNARE proteins, and F-actin remodeling in insulin secretion. Thus, my *central hypothesis* is that, in the pancreatic beta cell, glucose signals through a Cdc42-PAK1-mediated pathway to remodel the F-actin cytoskeleton to mobilize insulin granules to SNARE docking sites at the PM to evoke glucose stimulated second phase insulin secretion. To test this hypothesis, two main Aims were pursued: 1) Determine the role and requirement for PAK1 signaling in F-actin remodeling, insulin granule mobilization, and SNARE docking at the PM in glucose-stimulated insulin secretion, and 2) Investigate the role for the novel Syntaxin 4-interacting protein Gelsolin in regulating glucose-stimulated insulin secretion, F-actin remodeling, and Syntaxin 4-F-actin dynamics.

**CHAPTER 2. GLUCOSE MEDIATES EFFECTS ON CORTIAL F-ACTIN
REMODELING AND AMPLIFICATION OF INSULIN SECRETION IN PANCREATIC
BETA CELLS VIA PAK1-MEK-ERK SIGNALING**

Some of the text in this Chapter will be submitted for publication:

Kalwat MA, Wang Z, Yoder SM, and Thurmond DC. Glucose mediates effects on F-actin dynamics and the MAP kinase pathway in pancreatic beta cells via PAK1.

Author Contributions: Wang Z generated data for figure 2-1A. Yoder SM provided reagents for figure 2-1C. The rest of the figures and text were generated by Kalwat MA.

2.1 INTRODUCTION

Insulin is secreted from pancreatic beta cells in a biphasic manner in response to fuel secretagogues. A first phase peak in secretion occurs within 10 minutes after glucose stimulation. Following first phase, a lower-rate sustained second-phase secretion occurs that can persist for hours (55). Biphasic insulin secretion occurs when glucose enters the beta cell through GLUT2 glucose transporters and is metabolized leading to an increased ATP:ADP ratio (55). This leads to the inhibition of ATP-sensitive K^+ channels, causing membrane depolarization and calcium influx through voltage-sensitive calcium channels which results in insulin granule exocytosis (26-29). Both phases of insulin exocytosis are facilitated by SNARE proteins. The target membrane-SNAREs Syntaxin 1 and 4 and SNAP-25 reside at the plasma membrane while the vesicle-SNARE VAMP2 is present on the insulin granule (194). Syntaxin, SNAP-25, and VAMP2 form a heterotrimeric complex to facilitate insulin exocytosis. Syntaxin 1 is only required for the first phase of secretion (42) while Syntaxin 4 is important for both phases (39), although the exact mechanisms by which Syntaxin 4 participates in each of these phases of insulin secretion are not fully understood.

Glucose signaling and activation of the Cdc42-PAK1 pathway is required for second phase insulin secretion and may be a potential link to SNARE-regulated biphasic insulin secretion since Cdc42 can interact with VAMP2 (43, 45, 86). Cdc42-PAK1 signaling has been elucidated as a key proximal step leading to second-phase glucose-stimulated insulin secretion since the depletion or inactivation of either of these proteins selectively impairs the second-phase (43, 45). Multiple studies have shown that upon entry of stimulatory levels of glucose into the beta cell, Cdc42 is activated within about 3 min (43, 65, 74, 85), concurrent with the first phase release of insulin. Following Cdc42 activation, PAK1 becomes phosphorylated and activated in a manner dependent on Cdc42 within 5-10 min more of glucose stimulation (43). Concomitant with Cdc42-PAK1 pathway activation, glucose stimulates cortical F-actin remodeling after 5-10

minutes of stimulation (33, 65, 143, 172). Cortical F-actin remodeling is characterized by a diminished F-actin intensity or breaking up of the cortical ring of F-actin (33). By 20 min Rac1 becomes activated and this is dependent on PAK1 activation (43). Thus while Rac1 is a known actin remodeling factor, its activation would appear to occur too late to account for the initialization of F-actin remodeling induced by glucose stimulation. Demonstrations of why regulated F-actin remodeling is important for secretion come from studies where F-actin is disrupted or stabilized pharmacologically in beta cells, leading to potentiated albeit dysregulated biphasic insulin secretion (33, 34). Experimental F-actin disruption is normally done using the drug latrunculin which specifically binds to G-actin and sequesters it preventing polymerization (207). Latrunculin treatment of beta cells causes the insulin granules to accumulate at the plasma membrane, but does not raise basal insulin secretion (64). Upon stimulation with glucose, secretion is elevated many fold above normal and there is no distinction between first and second phase insulin secretion (33). In the case of F-actin stabilization, the drug jasplakinolide is normally used, although care must be taken in data interpretation due to the effects of jasplakinolide. Jasplakinolide causes the nucleation of new F-actin in addition to stabilizing the F-actin (207). Since Jasplakinolide does not simply prevent the remodeling of the F-actin structure, this drug cannot answer the question about whether prevention of F-actin remodeling would prohibit insulin secretion. The effects of latrunculin on beta cells suggest that F-actin may be a barrier under unstimulated conditions, and the similar effects of jasplakinolide suggest F-actin may also have an active role in promoting exocytosis.

In considering the molecular mechanism(s) by which glucose signals to initiate F-actin remodeling in beta cells, PAK1 emerges as a logical candidate to couple signaling to F-actin changes. For example, PAK1 has multiple substrates with the potential to elicit changes in F-actin structure, such as LIMK (89), Filamin A (90), p41-Arc (93), MLCK-MLC pathways (91), and the MAPK pathway through Raf/MEK (87, 92). As a scaffold, PAK1 interacts with proteins such as the small GTPases Rac1 and Cdc42, as well as the beta cell guanine nucleotide exchange

factor for Cdc42, β Pix/Cool-1 (73). PAK1 is implicated in type 2 diabetes, since islets from type 2 diabetic individuals contain 80% less PAK1 protein than normal individuals (43). Moreover, PAK1 is located in a region of chromosome 11 that is linked to type 2 diabetes disease risk (208, 209), adding the potential for PAK1 gene variants to contribute to disease. Therefore, determining how PAK1 functions in regulating insulin secretory processes in islet beta cells may provide insight into why defects in PAK1 are linked to diabetes or diabetic susceptibility. Since our current understanding of both the regulation of second phase insulin secretion and glucose-stimulated F-actin remodeling is incomplete, understanding how glucose mediates these processes is critical to the advancement of our knowledge of beta cell biology and to elucidate potential therapeutic avenues for diabetes. From a broader perspective, since PAK1 is a ubiquitous protein, our results have relevance to other cell systems wherein PAK1 regulates secretory processes such as GLUT4 vesicle exocytosis in skeletal muscle (45).

In this study we demonstrate the requirement of PAK1 signaling for normal glucose-induced F-actin remodeling and implicate PAK1 in SNARE-mediated exocytosis. Blocking PAK1 activation using the specific inhibitor IPA3 prevented normal glucose-induced F-actin remodeling. IPA3 treatment also prevented the translocation of VAMP2-bound insulin granules to the plasma membrane. In the beta cell, PAK1 was shown to signal through the MEK-ERK pathway in response to glucose to elicit cortical F-actin remodeling. Interestingly, PAK1 inhibition also resulted in altered Syntaxin 4-F-actin complexation. Since Syntaxin 4-F-actin interactions are known to control insulin granule docking/fusion at the PM, disruption of this by IPA3 or PAK1 depletion may account for the selective attenuation of second phase insulin release. These results support a model whereby glucose-stimulated Cdc42-PAK1-MEK/ERK signaling leads to F-actin remodeling, perhaps to direct insulin granule translocation to Syntaxin 4 SNARE docking sites at the plasma membrane to support insulin secretion.

2.2 MATERIALS AND METHODS

2.2.1 Materials, Reagents, and Plasmids

Rhodamine-Phalloidin was from Life Technologies (Grand Island, NY). Latrunculin B was from Calbiochem. Rabbit anti-phospho-ERK1/2, mouse anti-ERK, rabbit anti-phospho-MEK, rabbit anti-MEK, rabbit anti-PAK1, rabbit anti-phospho-Filamin A (Ser2152), and rabbit anti-Filamin A antibodies were from Cell Signaling Technology (Danvers, MA). Diazoxide, IPA3, and rabbit anti-phospho-PAK1 (Thr423) were from Santa Cruz (Santa Cruz, CA). Rabbit anti-actin was from Sigma (St. Louis, MO). Two rabbit polyclonal anti-Syntaxin 4 antibodies were obtained for use in coimmunoprecipitation (Chemicon, Temecula, CA) and for immunoblotting (antibody described in (210)). Mouse anti-SNAP-25 was from BD Biosciences (Mountain View, CA). Mouse anti-VAMP2 was from Synaptic Systems (Germany). Goat anti-mouse horseradish peroxidase secondary antibody and PD0325901 were obtained from Thermo Fisher Scientific (Rockford, IL). Goat anti-rabbit horseradish peroxidase secondary antibody and TransFectin lipid reagent were acquired from Bio-Rad (Hercules, CA). ECL reagent and Supersignal Femto were purchased from GE Healthcare (Piscataway, NJ) and Pierce, respectively. The rat insulin radioimmunoassay kits were obtained from Millipore (Billerica, MA). The Lifeact-GFP plasmid was kindly provided by Dr. Louis Philippon and was generated as described (211). The F/G Actin ratio kit was obtained from Cytoskeleton (Denver, CO).

2.2.2 Cell Culture, transient transfection, and secretion assays

MIN6 beta cells (a gift from Dr. John Hutton, University of Colorado Health Sciences Center, Denver, CO) were cultured in Dulbecco's modified Eagle's medium (25 mM glucose), supplemented with 15% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, 292 µg/ml L-glutamine, and 50 µM β-mercaptoethanol as described previously (201). MIN6 cells were transfected with Transfectin (Bio-Rad) according to the manufacturer's instructions and

cultured 48 h before use in experiments. For experiments where cells were starved and stimulated, MIN6 cells were washed twice with and incubated for 2 h in freshly prepared modified Krebs-Ringer bicarbonate buffer (MKRBB: 5 mM KCl, 120 mM NaCl, 15 mM HEPES, pH 7.4, 24 mM NaHCO₃, 1 mM MgCl₂, 2 mM CaCl₂, and 1 mg/ml radioimmunoassay-grade BSA). Cells were stimulated with 20 mM glucose as indicated and insulin secreted into the buffer was quantitated using a rat insulin radioimmunoassay kit (EMD Millipore, Billerica, MA). Cells were lysed in 1% Nonidet P-40 (NP-40) lysis buffer (25 mM HEPES, pH 7.4, 1% Nonidet P-40, 10% glycerol, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 137 mM NaCl, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 1 µg/ml pepstatin, 5 µg/ml leupeptin) and cleared of insoluble material by centrifugation for 10 min at 4°C for subsequent use in co-immunoprecipitation experiments.

2.2.3 Subcellular Fractionation

As described previously (74), all fractionation steps were performed at 4°C. Briefly, MIN6 cells at 70-80% confluence were harvested into 1 ml of homogenization buffer (20 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 250 mM sucrose, 1 mM dithiothreitol, 100 µM phenylmethylsulfonyl fluoride, 4 µg/ml aprotinin, 2 µg/ml pepstatin, and 10 µg/ml leupeptin). Cells were homogenized by 10 strokes through a 27-gauge needle, and then centrifuged at 900 x g for 10 min. Post-nuclear supernatants were centrifuged for 5500 x g for 15 min. The resulting supernatant was centrifuged at 25,000 x g for 20 min to obtain the secretory granule fraction in the pellet. This supernatant was centrifuged further at 100,000 x g for 1 h to acquire the cytosolic fraction. The PM fraction was prepared by mixing the post-nuclear pellet with 1 volume of Buffer A (0.25 M sucrose, 1 mM MgCl₂, and 10 mM Tris-HCl, pH 7.4) and 2 volumes of Buffer B (2 M sucrose, 1 mM MgCl₂, 10 mM Tris-HCl, pH 7.4). This mixture was overlaid with Buffer A and centrifuged at 113,000 x g for 1 h to obtain an interface containing the PM. The interface was collected, diluted to 1.5 ml with homogenization buffer, and centrifuged at 6000 x g for 10

min. The resulting pellet contained the PM fraction. All collected pellets were resuspended in 1% NP-40 lysis buffer.

2.2.4 Co-immunoprecipitation and Immunoblotting

For immunoprecipitation, 2-3 mg of cleared detergent lysate protein was combined with 1 µg of antibody per mg protein and the reaction rotated for 2 h at 4°C. Protein G Plus agarose beads (Santa Cruz) were added and reactions rotated at 4°C for an additional 2 h. Beads were pelleted and washed three times with lysis buffer and resulting immunoprecipitates were resolved on 10-12% SDS-PAGE and transfer to PVDF membranes for immunoblotting. Immunoreactive bands were visualized with ECL, ECL Prime (GE Healthcare, Piscataway, NJ), or Supersignal Femto (Pierce) reagents and imaged using a Chemi-Doc gel documentation system (Bio-Rad, Hercules, CA). Phosphorylated and total ERK blots were visualized using goat anti-mouse 680 and goat anti-rabbit 800 simultaneously and imaged on a Licor imaging system.

2.2.5 Immunofluorescence, confocal microscopy and scoring F-actin remodeling

MIN6 cells plated onto glass coverslips at 30% confluence were transiently transfected with 4 µg of plasmid DNA/35 mm well. After 48 h incubation, cells were placed in MKRBB for 2 h, followed by stimulation with 20 mM glucose for 5 min and then immediately fixed and permeabilized in fixation/permeabilization buffer (4% paraformaldehyde, 0.1% Triton X-100 at 4°C) for 10 min in the dark. Fixed and permeabilized cells were blocked in blocking buffer (1% BSA plus 5% donkey serum in PBS) for 1 h at room temperature, followed by incubation with 0.17 µM Rhodamine-Phalloidin for 1 h, per manufacturer instructions. MIN6 cells were then washed three times with phosphate-buffered saline (pH 7.4). During the final wash, DAPI was added to stain nuclei. All cells were washed again with PBS, and mounted (using Vectashield) for confocal fluorescence microscopy. Fluorescent cells were imaged using single-channel scanning with a 60X objective (2X zoom) using an Olympus FV1000-MPE confocal microscope

(Olympus, Center Valley, PA). To score F-actin remodeling, only cells on the outside of clusters were counted. Cells with discontinuous cortical F-actin after 5 min of glucose stimulation were scored as exhibiting F-actin remodeling.

2.2.6 Live-cell imaging

MIN6 beta cells were plated on 35 mm glass-bottom MatTek culture dishes, grown to 30-50% confluency and transfected with Lifeact-GFP. 48 h later cells were starved in MKRBB containing either DMSO or drugs as stated in figure legends. For confocal live-cell imaging, cells were constantly perfused with MKRBB containing DMSO or IPA3 and imaged on an Olympus FV1000-MPE. For Apotome live-cell imaging data was collected using a Zeiss Axio Observer with a Plan-Apochromat 63X objective equipped with a Hamamatsu Orca-ER digital camera and an Apotome and analyzed using ImageJ software (NIH).

2.2.7 Mouse islet isolation, perfusion, and islet immunoblot analysis

All studies involving mice followed the Guidelines for the Use and Care of Laboratory Animals at Indiana University School of Medicine. Wild type male C57BL/6J mice were sacrificed for pancreatic islet isolation as previously described (212). Briefly, mouse islets were isolated from collagenase-perfused pancreata using a combination of purified collagenase (Czyme MA, VitaCyte, Indianapolis, IN) and neutral protease (Czyme BP Protease, VitaCyte). Islets were carefully handpicked and size-matched into batches of 50 islets per column for perfusion. Perfusion studies were carried out as previously described (200). Insulin secreted into the buffer and insulin content in the corresponding islet lysates was quantified by Rat Insulin RIA (Millipore). To prepare stimulated islet samples for Western analysis, 100-200 islets were incubated for 1 h in KRBH buffer (10 mM HEPES pH 7.4, 134 mM NaCl, 5 mM NaHCO₃, 4.8 mM KCl, 1 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄ containing 0.5 mg/ml BSA) supplemented with 2.8 mM glucose. Islets were then stimulated for 1 h in 16.7 mM glucose,

washed in cold PBS, and lysed and boiled in Laemmli sample buffer before SDS-PAGE and immunoblot analysis.

2.2.8 Human Islet Culture

Pancreatic human islets were obtained through the Integrated Islet Distribution Program, IIDP. Criteria for human donor islet acceptance: receipt within 36 h of isolation, and of at least 80% purity and 75% viability. Upon receipt, human islets were first allowed to recover in CMRL medium for 2 h, and then were handpicked using a green gelatin filter to eliminate residual non-islet material. Human islets were then used immediately for glucose time course experiments by preincubation in KRBH for 2 h, followed by stimulation with glucose for the times indicated in the figures. For IPA3 treatment, islets were cultured overnight in CMRL containing either DMSO or IPA3 (7.5 μ M).

2.2.9 Statistical Analysis

All quantified data are expressed as mean \pm S.E. Data were evaluated using Student's t test and considered significant if $P < 0.05$.

2.3 RESULTS

2.3.1 PAK1 is activated in human islets and is required for MEK activation

While it has been shown that PAK1 activation occurs in human islets and is dependent on upstream glucose-stimulated Cdc42 activation (45), whether the time course of activation in human islets mimics that observed in rodent cells was undetermined. Toward this, human islets were preincubated at 2.8 mM glucose followed by incubation in 16.7 mM glucose for either 5, 10, or 20 minutes and lysed for immunoblot analysis. We observed that PAK1 phosphorylation on Thr 423 was indeed increased by 5 min of glucose and was maintained at 10 and 20 min, similar to studies with mouse clonal MIN6 beta cells (Figure 2-1A). ERK phosphorylation served as an indicator of islet responsiveness to glucose and was increased similarly in response to 5 min glucose stimulation (Figure 2-1A). Furthermore, human islets that were pretreated overnight with the PAK1 inhibitor IPA3 exhibited reduced PAK1 phosphorylation on Thr 423 and reduced MEK phosphorylation on Ser 217/221 in response to glucose stimulation (Figure 2-1B), indicating that PAK1 signaling in human islets is required upstream for MEK activation. We confirmed these results in MIN6 cells showing that MEK is activated in response to glucose by 5 min (Figure 2-1C) and IPA3 can prevent MEK activation (Figure 2-1D). These data demonstrate that PAK1 is necessary for MEK activation in both human islets and MIN6 cells, and that the MIN6 cell line is a suitable model to study PAK1 signaling.

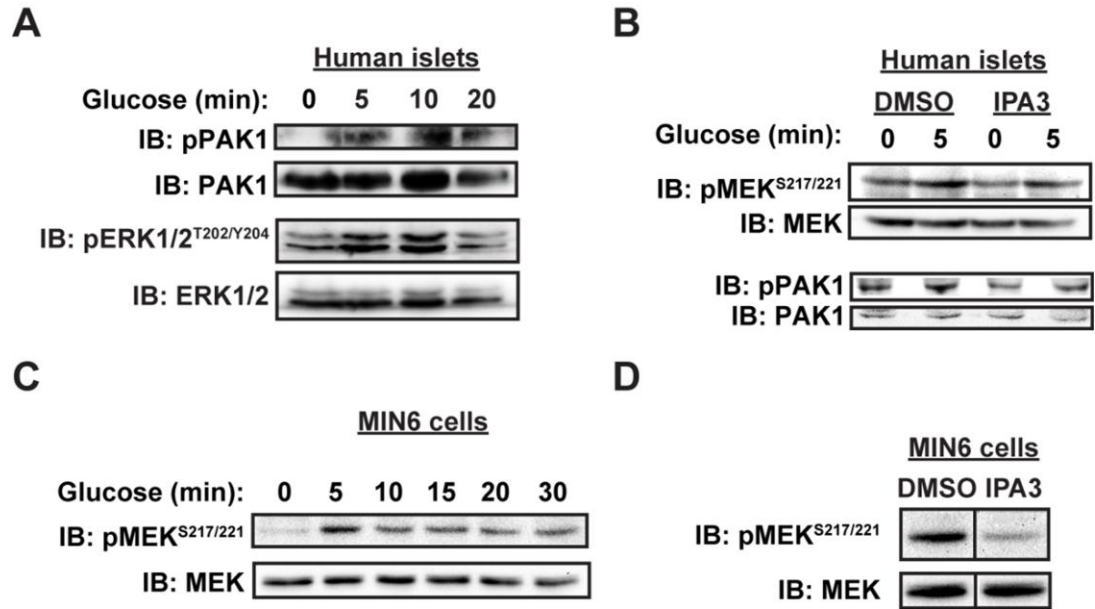


Figure 2-1. PAK1 is phosphorylated and is required for MEK activation in human islets. **A)** Human islets were incubated in KRBH with 2.8 mM glucose for 2 h and then stimulated with 16.7 mM glucose for 0, 5, 10, and 20 min. Islets were lysed for SDS-PAGE and immunoblotting for phosphorylated and total PAK1 and ERK1/2. Data are representative of at least three different batches of human islets. **B)** Human islets were incubated overnight 15h in CMRL with containing either vehicle (DMSO) or 7.5 μ M IPA3. Islets were then incubated in KRBH with 2.8 mM glucose for 2 h, still in the presence of vehicle or IPA3, and then stimulated with 16.7 mM glucose for 5 min before harvest for SDS-PAGE and immunoblotting for either phosphorylated (pMEK1/2) and total MEK1/2 or phosphorylated (pPAK1) and total PAK1. Blots shown for pPAK1/PAK and pMEK1/2/MEK1/2 are from different batches of islets. **C)** MIN6 cells were starved for 2 h in MKRBB before stimulation with 20 mM glucose for 0, 5, 10, 15, 20, and 30 min. Detergent whole cell lysates were collected and subjected to SDS-PAGE analysis and immunoblotting with antibodies to phosphorylated and total MEK1/2. **D)** MIN6 cells were treated as in (C) except the MKRBB contained either vehicle or 15 μ M IPA3 for 1 h prior to the 5 min glucose before stimulation. Data are representative of at least three independent experiments.

2.3.2 PAK1 activity is necessary for glucose-induced cortical F-actin remodeling

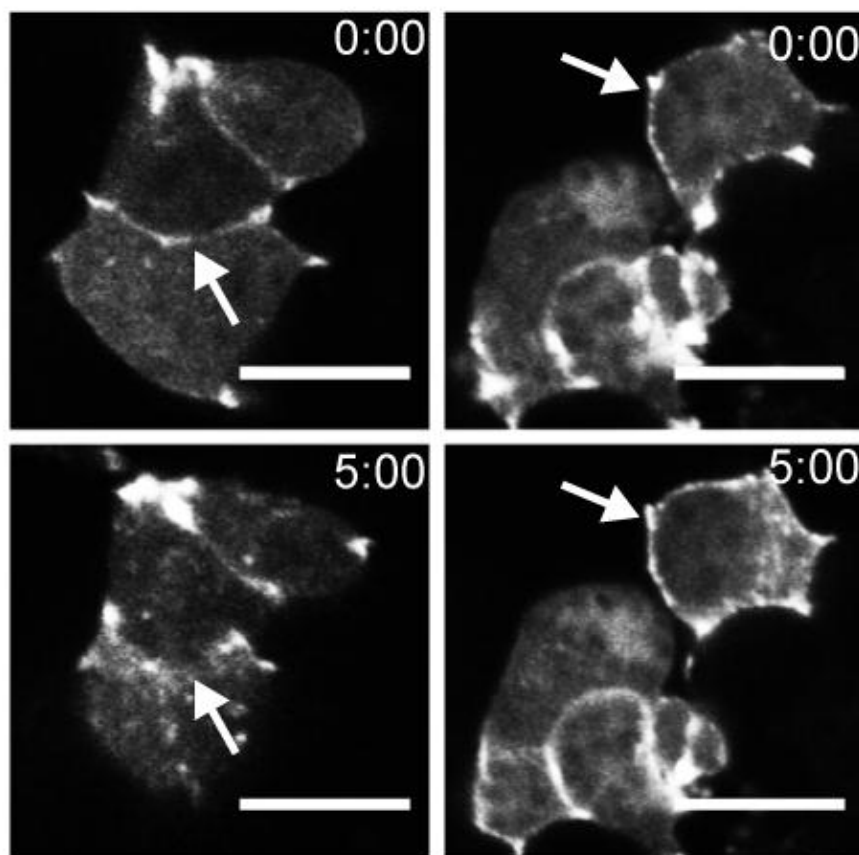
To determine whether PAK1 activation is required for changes in cortical F-actin remodeling in response to glucose, we used the Lifeact-GFP biosensor (213). Lifeact is a 17 residue peptide from the actin binding protein Abp140 linked N-terminally to GFP as a fusion protein that has been shown to bind specifically to F-actin in live cells without adversely affecting F-actin dynamics. Lifeact-GFP allows live cell imaging of F-actin dynamics, as previously demonstrated in beta cells (136, 211). Glucose stimulation elicited visible changes in the cortical F-actin structure in control (DMSO) treated cells (Figure 2-2A), consistent with previously published work showing that glucose stimulation causes F-actin remodeling in beta cells (33, 137, 143). In contrast, cells that had been preincubated for 10 min with the PAK1 inhibitor IPA3 prior to glucose stimulation showed no glucose-induced changes to cortical F-actin structure (Figure 2-2A). Treatment with IPA3 had no adverse effect on the overall F/G-actin ratio under either basal or glucose-stimulated conditions (Figure 2-2B), indicating that the effect of IPA3-mediated PAK1 inhibition on glucose-induced F-actin remodeling was not likely due to increased levels of cellular F-actin, but instead was suggestive of a block in glucose-induced actin remodeling.

A

Live-cell imaging
Lifeact-GFP MIN6

DMSO

IPA3



B

F/G-Actin Ratio Assay



Figure 2-2. PAK1 activity is required for glucose-stimulated cortical F-actin remodeling in MIN6 beta cells. **A)** MIN6 cells were transfected with Lifeact-GFP plasmid DNA and incubated to allow protein expression for 48 h. Lifeact-GFP expressing cells were then preincubated for 2 h in MKRBB, and either vehicle (DMSO) or 30 μ M IPA3 was added to the buffer 10 min before live-cell confocal imaging. Images were collected every minute for 5 min. Representative images of 11 cells from 3 independent experiments assessing basal (0:00) and stimulated (5:00) Lifeact-GFP in MIN6 cells are shown. *Scale bar*, 10 μ m. **B)** MIN6 cells were preincubated in MKRBB for 2 h followed by a 10 min pretreatment with either DMSO or 30 μ M IPA3. Cells were harvested for F/G-Actin ratio analysis (F/G-actin ratio kit, Cytoskeleton, Inc). Samples were subjected to SDS-PAGE and actin immunoblotting (IB). S, supernatant (G-actin). P, pellet (F-actin). Data are representative of three independent experiments.

2.3.3 PAK1 activity is required for VAMP2-bound insulin granule accumulation at the PM in response to glucose

While PAK1 is known to be required for second-phase insulin secretion, the exact mechanism(s) or downstream signaling pathway(s) which require PAK1 remained unclear. PAK1 may be required for granule recruitment to the PM, increasing the release competency of granules at the PM, or both. We used subcellular fractionation of MIN6 beta cells to address whether PAK1 is required for VAMP2 accumulation at the PM during glucose stimulation. VAMP2 is the v-SNARE protein present on the insulin granule and known to be required for SNARE mediated insulin exocytosis (214), and therefore serves as a marker of insulin granule locale within the beta cell. Since PAK1 is required for second phase insulin secretion, 20 min as opposed to 5 min of glucose stimulation was used to assay the impact of PAK1 inhibition on VAMP2 accumulation at the PM. Compared to unstimulated cells, the ratio of VAMP2 between the PM and SG (secretory granule) fractions was significantly increased by ~40% after 20 min of glucose-stimulation, consistent with previous studies (Figure 2-3) (86). However, cells treated with IPA3 followed by 20 min of glucose stimulation exhibited no increase in the VAMP2 PM/SG ratio (Figure 2-3). This data suggested that PAK1 activity may be required for insulin granule recruitment to and/or retention/capture at the PM.

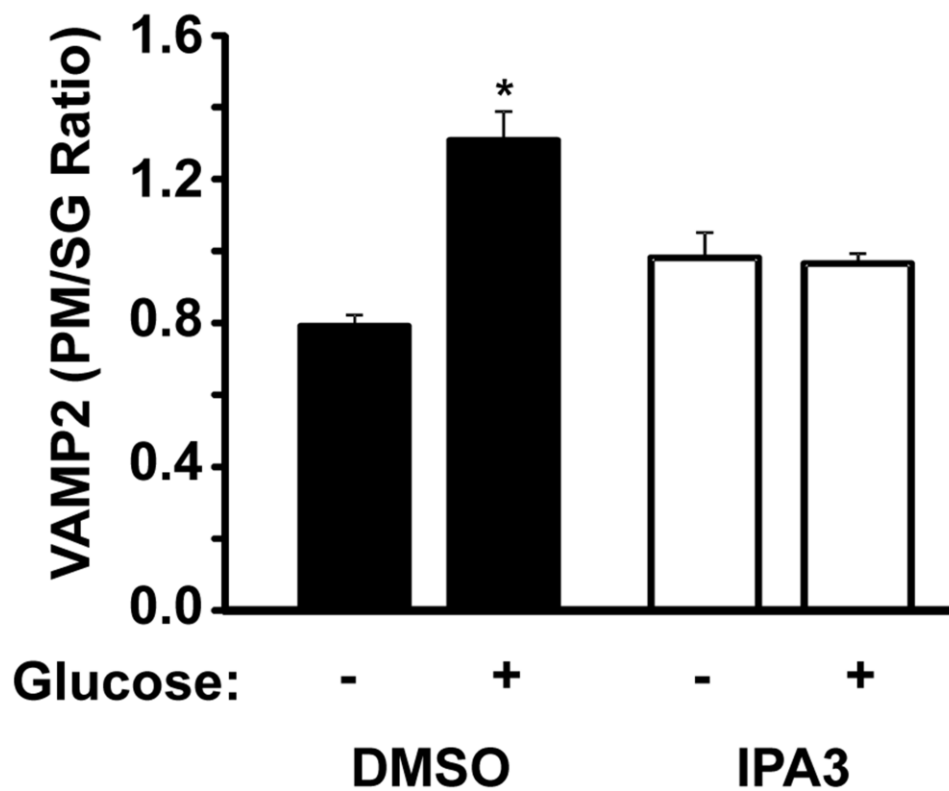


Figure 2-3. PAK1 is required for glucose-induced VAMP2 accumulation at the plasma membrane. MIN6 cells were preincubated for 2 h in MKRBB and either vehicle (DMSO) or 30 μ M IPA3 was added 10 min prior to stimulation with 20 mM glucose for 20 min. Cells were harvested and subjected to subcellular fractionation to isolate plasma membrane (PM) and secretory granule (SG) fractions. Samples from the PM and SG of stimulated and unstimulated cells were analyzed by SDS-PAGE and immunoblotting and quantitation of VAMP2 by densitometry. The amount of VAMP2 in the PM divided by the amount of VAMP2 in the SG yielded the VAMP2 PM/SG ratio which increased in response to glucose in control cells. The bar graph represents the average \pm S.E. for three independent experiments (*, $P < 0.05$, versus unstimulated DMSO).

2.3.4 ERK signaling contributes to the amplifying pathway of insulin secretion and cortical F-actin remodeling

Having established PAK1 as an upstream signal required for MEK1/2 and ERK1/2 activation, we next questioned whether PAK1-MEK-ERK signaling accounted for PAK1's requirement in glucose-stimulated insulin secretion. To test this, we monitored insulin secretion from MIN6 cells treated with the MEK inhibitor, PD03, to prevent ERK activation. PD03 has a much lower IC₅₀ than its predecessor MEK inhibitors PD98059 or U0126, and has very little activity against kinases other than MEK, allowing its use at lower concentrations that reduce the risk of off-target effects (181). Low dosage PD03 treatment was first verified to block ERK activation by immunoblot analysis (Figure 2-4A), and was subsequently found to result in a 30% reduction in glucose-stimulated insulin secretion after 30 min of stimulation (Figure 2-4B). Since a 30 min glucose stimulation will encompass insulin release during both phases, we next employed the diazoxide paradigm in an effort to examine the requirement for ERK in the amplifying phase of insulin secretion in MIN6 cells. MIN6 cells were pretreated with either vehicle (DMSO) or PD03 for 2 h, followed by a 10 min pretreatment with diazoxide before stimulation with 30 mM KCl or 30 mM KCl plus 20 mM glucose. Control cells showed a robust 3-fold glucose-induced amplification response during the 30 min stimulation period (Figure 2-4C). By contrast, PD03-treatment caused a 30% reduction in glucose-amplified insulin secretion (Figure 2-4C). In these cells, ERK activation was initiated by the triggering pathway conditions, but was heightened by the addition of glucose to activate the amplification pathway (Figure 2D). PD03 efficiently blocked ERK activation under all treatment conditions (Figure 2-4D). To determine whether ERK's role downstream of PAK1 in the amplifying pathway involved effects on cortical F-actin remodeling, MIN6 cells were pretreated with vehicle (DMSO) or PD03 for 2 h followed by a 5 min stimulation with glucose. Control cells exhibited remodeling of F-actin after 5 min of glucose stimulation, while the F-actin remodeling in PD03-treated cells was blunted (Figure 2-4E). IPA3 treated cells served as a control and showed the expected blunting of

glucose-induced cortical F-actin remodeling (Figure 2-4E). Given that PD03 efficiently blocked all ERK activity yet some insulin release attributable to the amplification was retained, it remains possible that PAK1-MEK-ERK may not be the exclusive signaling cascade to account for the amplification pathway of insulin secretion.

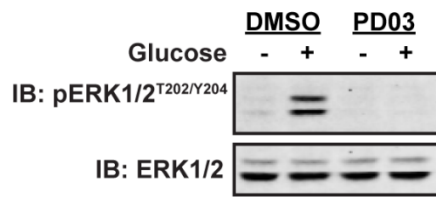
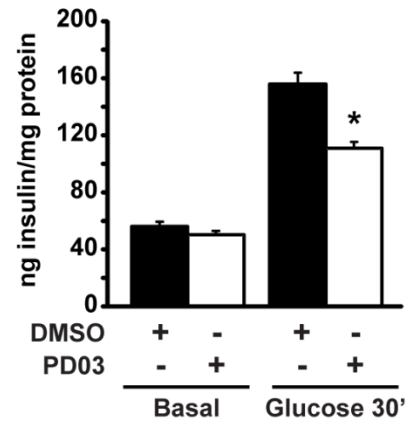
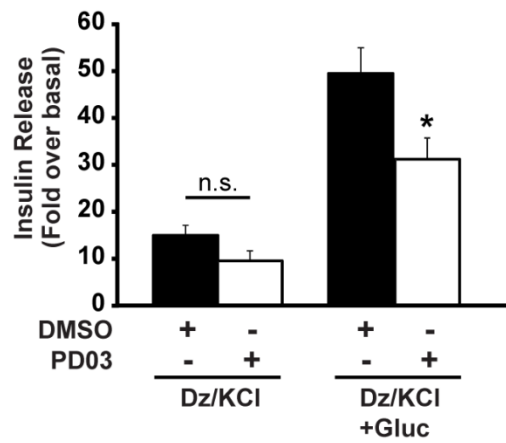
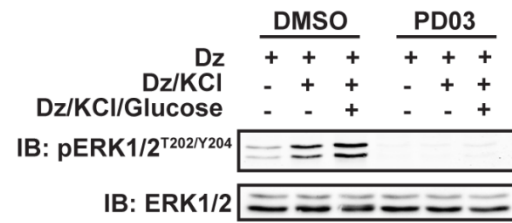
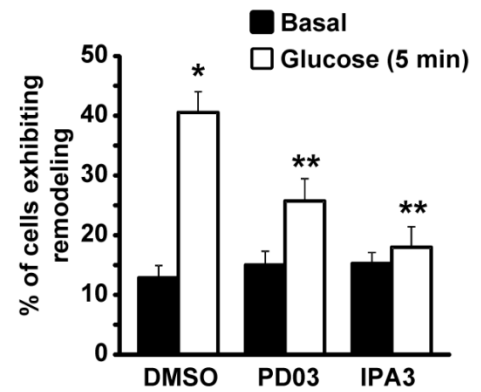
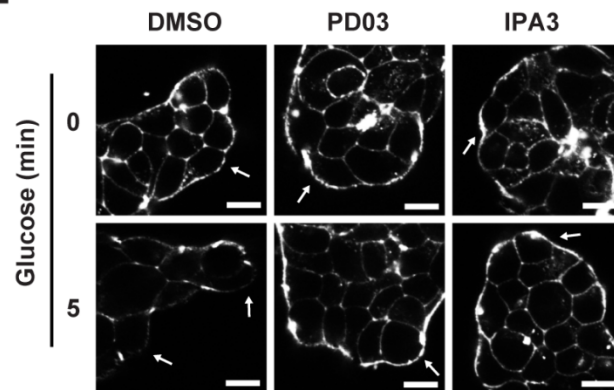
A**B****C****D****E**

Figure 2-4. ERK signaling contributes to the amplifying pathway and F-actin remodeling. **A)** MIN6 cells were preincubated for 2 h in MKRBB containing either vehicle (DMSO) or 1 μ M PD03 and then stimulated with 20 mM glucose for 30 min. Lysates were subjected to SDS-PAGE and immunoblotting for phosphorylated and total ERK1/2. **B)** Insulin secreted into the MKRBB from MIN6 cells in (A) was measured. Bars represents the mean \pm S.E. of at least three independent experiments from (C) (*, $P < 0.05$, *versus* DMSO). **C)** MIN6 cells treated as in (A) were exposed to 250 mM diazoxide (Dz) for 10 min before stimulation with 35 mM KCl or 35 mM KCl plus 20 mM glucose for 30 min. Supernatants were collected for insulin radioimmunoassay. Bar graph represents the mean \pm S.E. of at least three independent experiments (*, $P < 0.05$, *versus* DMSO). **D)** Lysates from (C) were subjected to SDS-PAGE and immunoblot (IB) analysis for phosphorylated and total ERK1/2. **E)** MIN6 cells plated on glass cover slides were pre-incubated in MKRBB for 2 h and left unstimulated (top panels) or stimulated with 20 mM glucose for 5 min (bottom panels). Cells were fixed, permeabilized and cortical F-actin detected by Rhodamine-Phalloidin staining. Cells were imaged at the midplane by single channel scanning confocal microscopy to determine F-actin remodeling. Arrows indicate cortical regions of MIN6 cells. *Scale bar*, 10 μ m. Bar graph represents the mean % cells exhibiting F-actin remodeling \pm S.E. of three independent experiments where each experiment contained 6 to 12 fields of cells. (*, $P < 0.05$, stimulated DMSO *versus* unstimulated DMSO, ** $P < 0.05$, *versus* stimulated DMSO).

2.3.5 ERK activation is important for insulin secretion in mouse islets

A central tenet regarding the importance of second phase insulin secretion purports that granules mobilized towards the PM during the end of this phase serve as the pool of granules released in the subsequent round of GSIS (23, 32). To determine the requirement for ERK activity in this paradigm we perfused islets pre-treated with PD03 for 30 min through a full round of first and second phase which was normal (data not shown), rested the islets for 20 min at 2.8 mM glucose still in the presence of DMSO or PD03, and then subjected them to a subsequent round of stimulation using 30 mM KCl (Figure 2-5A). KCl is known to elicit rapid exocytosis of granules pre-docked at the PM (2). Compared with control vehicle treated islets, the PD03-treated islets secreted ~50% less insulin in response to KCl stimulation (Figure 2-5B, C). The ability of PD03 to prevent ERK activation was confirmed in islets subjected to this treatment paradigm (Figure 2-5C). Islet insulin content was unchanged by PD03 treatment (Figure 2-5D). These data may implicate ERK in the refilling of the readily releasable pool after a glucose challenge, and are consistent with the requirement for PAK1 signaling to ERK to elicit second-phase insulin secretion.

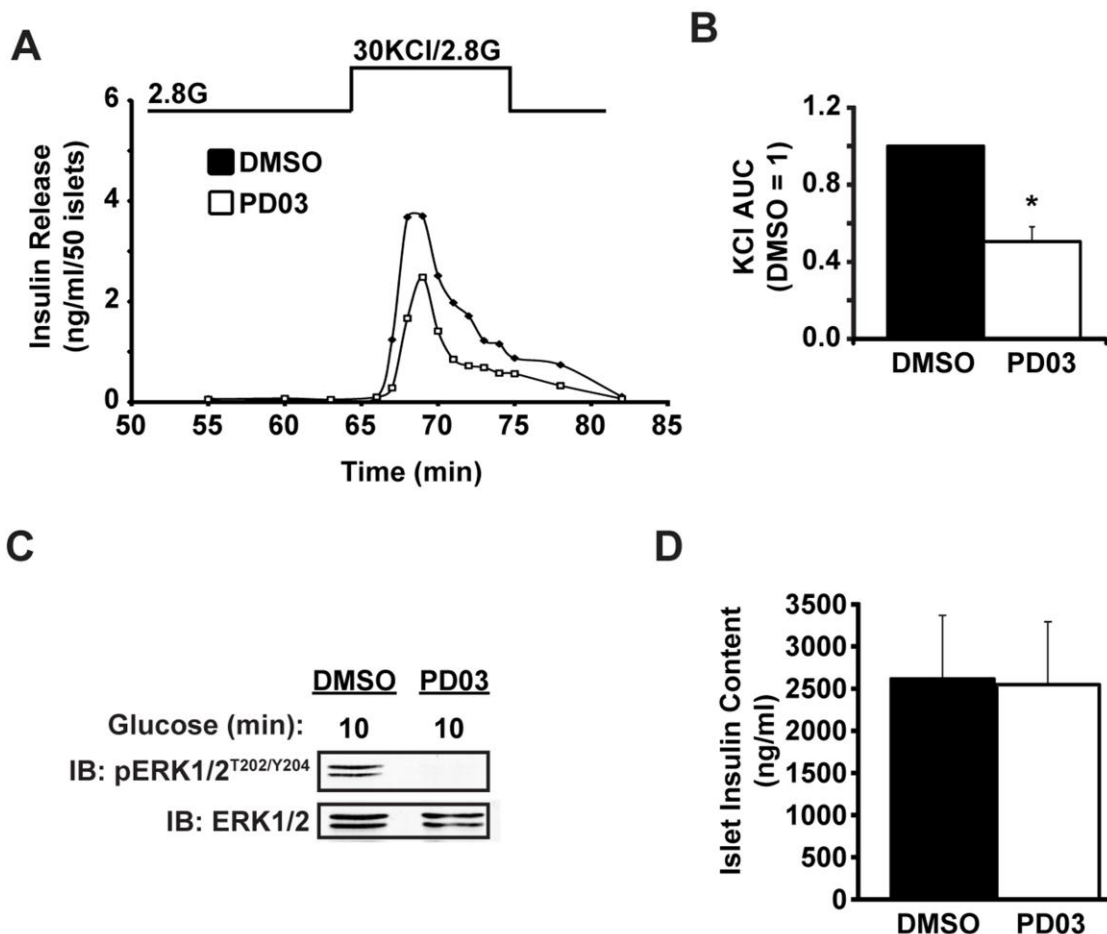


Figure 2-5. ERK signaling is required for insulin secretion after a repeated stimulation. **A)** 50 size-matched mouse islets were perfused in KRBH in the constant presence of either vehicle (DMSO) or 1 μ M PD03. Islets were first perfused under 2.8 mM glucose for 30 min, then 16.7 mM glucose for 35 min, followed by a 20 min rest period at 2.8 mM glucose. The islets were then stimulated with 35 mM KCl and 2.8 mM glucose for 10 min. Insulin content of the perfusate and the islets was determined by insulin radioimmunoassay. Trace is the average of three independent experiments. **B)** Bar graph represents the mean area under the curve (AUC) normalized to DMSO treatment \pm S.E. of three independent experiments from (A). AUC was calculated from the 10 min duration of KCl stimulation (66 to 75 min) (*, $P < 0.05$, versus DMSO). **C)** Mouse islets were preincubated in KRBH with 2.8 mM glucose in the presence of either DMSO or 1 μ M PD03 for 1 h followed by stimulation with 16.7 mM glucose for 10 min. Islets were lysed and subjected to SDS-PAGE and immunoblotting for phosphorylated and total ERK1/2. **D)** Insulin content was measured in islets from perfusion experiments in (A) with an insulin RIA kit. Bar graph represents the mean \pm S.E. of three independent experiments.

2.3.6 PAK1 signaling is coupled to F-actin-Syntaxin 4 interactions

Since PAK1 impacts glucose-induced F-actin remodeling and is required for second phase insulin secretion, we investigated how PAK1 might affect the t-SNARE known to directly interact with F-actin, Syntaxin 4. Syntaxin 4 is an important SNARE protein since it is required for both phases of insulin secretion and is the only Syntaxin operative in second phase (39). Syntaxin 4-F-actin complexes exist under unstimulated conditions and dissociate after 5 min of glucose stimulation (64). At this same time, PAK1 is activated and F-actin remodeling is underway (33, 43). To investigate whether PAK1 signaling was coupled to Syntaxin 4-F-actin interactions, PAK1 activation was inhibited with IPA-3 in MIN6 cells and the cell lysates were used in Syntaxin 4 coimmunoprecipitation experiments. Under control conditions, F-actin was found to associate with Syntaxin 4 (Syntaxin 4 binds only to F- and not to G-actin (64)), and dissociated after 5 min of glucose stimulation (Figure 2-6), consistent with previous findings (64) and with the timing and occurrence of glucose-induced actin remodeling in beta cells. However, IPA-3 treatment resulted in F-actin dissociation from Syntaxin 4 under basal conditions, and the Syntaxin 4-F-actin complexes were further unresponsive to glucose stimulation (Figure 2-6). Unlike F-actin, SNAP-25 binding to Syntaxin 4 was similar under all conditions, suggesting a specific effect of IPA3-mediated inhibition of PAK1 on Syntaxin 4-F-actin complexes. These data suggest that glucose-induced PAK1 signaling is important for the normal cycling of Syntaxin 4-F-actin association/dissociation.

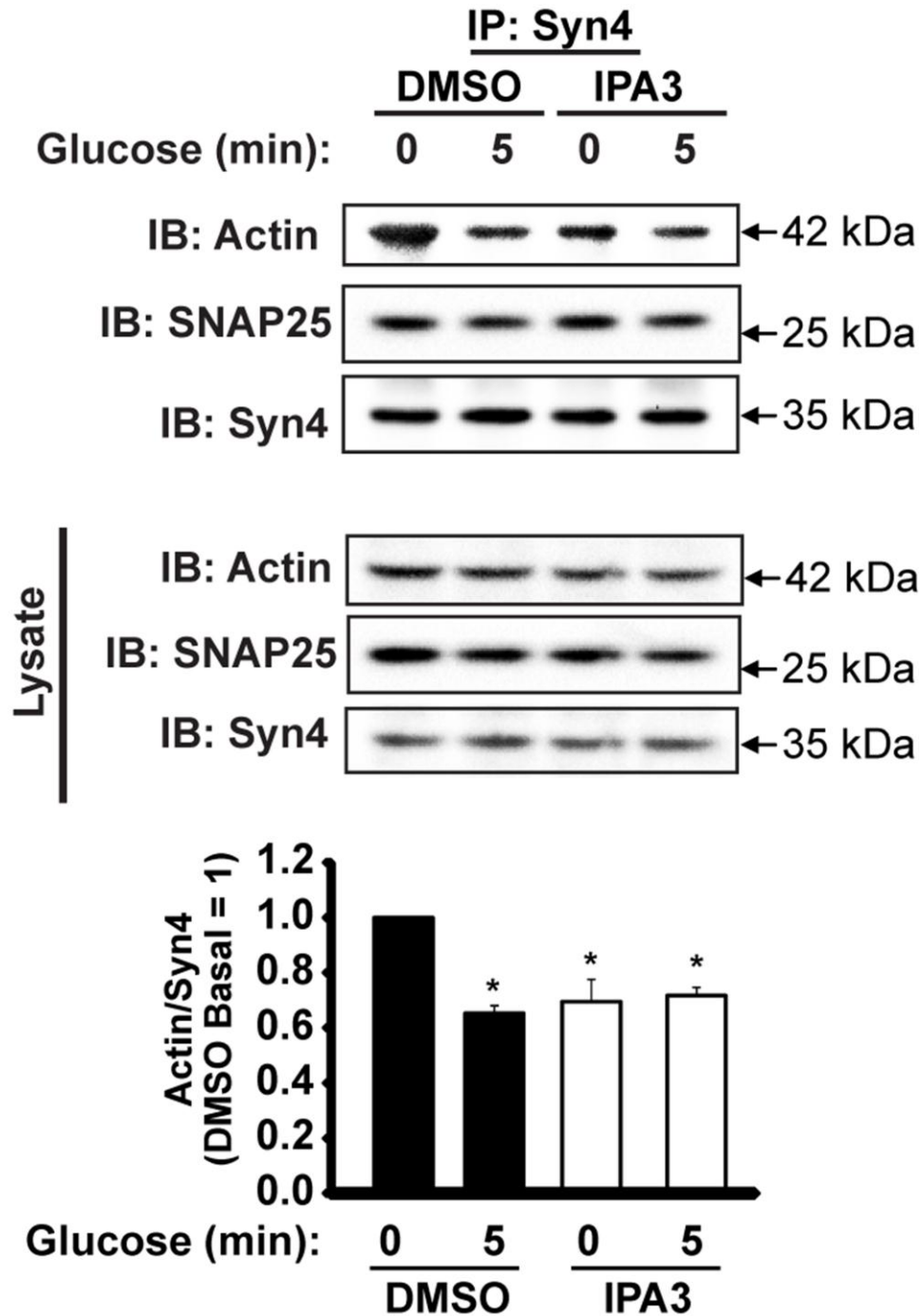


Figure 2-6. PAK1 signaling is linked to Syntaxin 4-F-actin complex regulation. MIN6 cells were preincubated in MKRBB for 2 h and then treated with either vehicle (DMSO) or 30 μ M IPA3 for 10 min before stimulation with 20 mM glucose for 5 min. Detergent lysates were collected and used in anti-Syntaxin 4 (Syn4) immunoprecipitation (IP) experiments. Association of SNAP-25 and actin was determined by immunoblotting (IB). Band intensities were quantitated by densitometry and expressed as the ratio of actin to Syn4 (normalized, basal ratio = 1 for each experiment). Bars represent the mean \pm S.E. of the three independent experiments (*, $P < 0.05$, versus unstimulated DMSO).

2.4 DISCUSSION

While PAK1 is required in the second phase of glucose-stimulated insulin secretion, how PAK1 is acting to carry out this task was unclear. Here we establish an importance for PAK1 signaling to F-actin cytoskeletal remodeling to facilitate glucose-stimulated insulin secretion. By use of kinase-selective inhibitors we mapped for the first time the signaling itinerary of PAK1 in the islet beta cell: PAK1-MEK-ERK. Moreover, each step was shown to be important for GSIS, specifically for the amplification pathway, suggesting this to be a linear cascade of events. PAK1-MEK-ERK signaling was also demonstrated to be critical for normal glucose-induced F-actin remodeling. In addition to its role in overall F-actin remodeling, PAK1 activation was implicated in the normal glucose-induced cycling of Syntaxin 4-F-actin association/dissociation. Since PAK1 activation was also required for VAMP2 accumulation at the PM, taken together these data are suggestive of a proximal role for PAK1 in a signaling pathway which leads downstream to F-actin-mediated trafficking and SNARE docking of insulin granules at the PM to elicit sustained exocytosis as shown in the model in Figure 2-7.

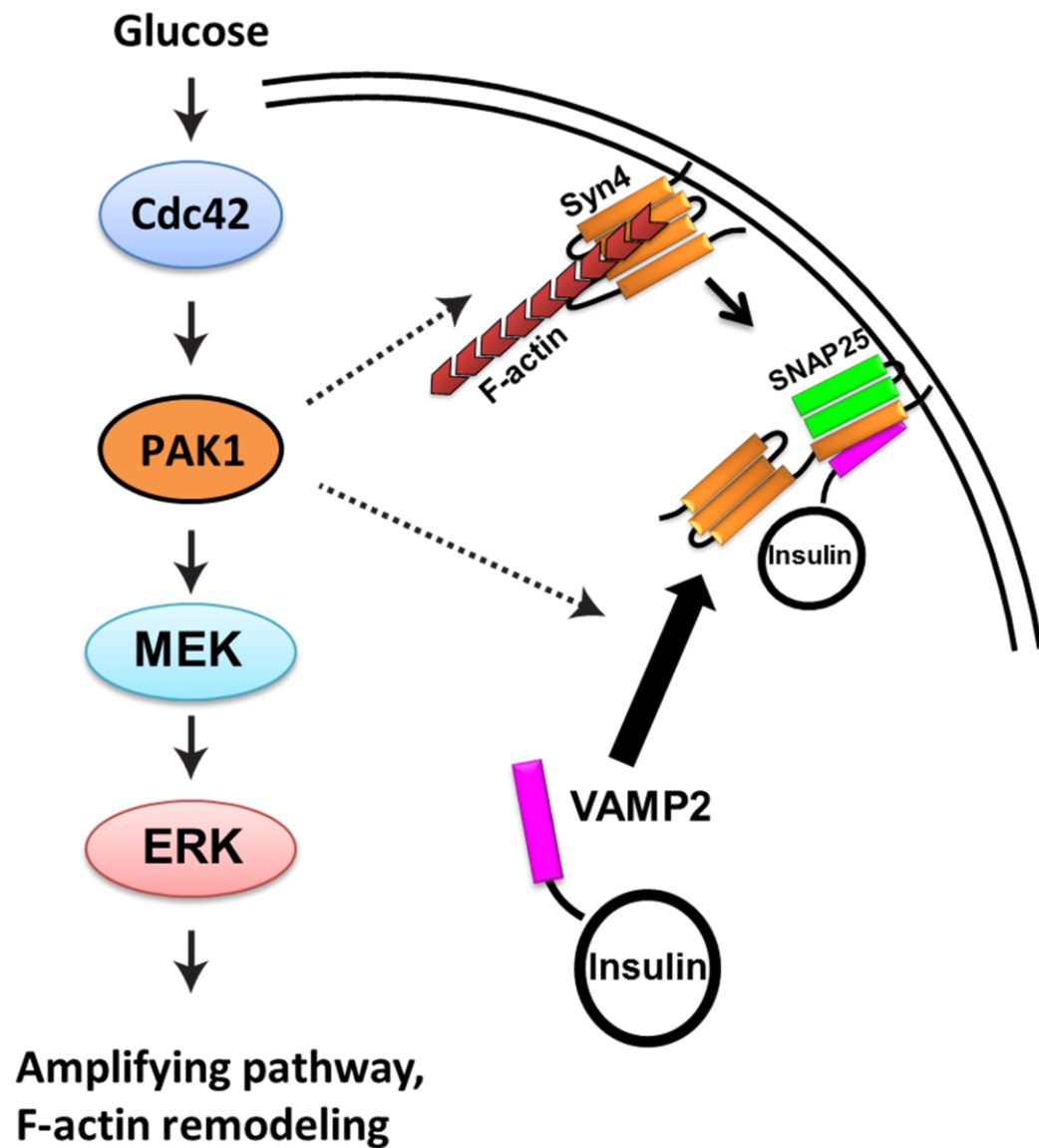


Figure 2-7. Model of Cdc42-PAK1 signaling in the beta cell. Glucose enters the beta cell, activating Cdc42 and PAK1. PAK1 signaling then regulates Syntaxin 4-F-actin complexes, VAMP2 accumulation at the plasma membrane, and MEK-ERK signaling to regulate amplification of insulin secretion and F-actin remodeling. Syn4, Syntaxin 4.

Glucose-stimulated activation of ERK lies downstream of PAK and MEK in the beta cell, but MEK activation is canonically regulated by the Raf kinases (173). Additionally, both C-Raf (also called Raf-1) and B-Raf have been investigated in beta cells for roles in GSIS (95, 215). B-Raf, but not C-Raf was activated by glucose in MIN6 cells (215), while in INS-1 832/13 cells use of the C-Raf inhibitor GW-5074 prevented ERK and Rac activation and inhibited GSIS (95). It is suggested that B-Raf activation may have a role early in glucose-stimulated ERK activation (from 5 to 15 min post-stimulation) while C-Raf may have a role at later time points (1 h post-stimulation) (215). PAK1 has been shown to phosphorylate both MEK and C-Raf (87, 175, 176) and constitutively active PAK1 decreased auto-inhibition of B-Raf (216). Our data combined with these observations suggest that in addition to direct activation of MEK, PAK1 may act through B-Raf/C-Raf to activate MEK and ERK in response to glucose. Since ERK activation was important for glucose-stimulated F-actin remodeling, future investigation will be required to elucidate the downstream effectors of ERK that mediate this effect.

Our results contribute to a growing body of data supportive of a role for ERK activation in the amplifying pathway. The existing data are a combination of findings using siRNA-mediated knockdown of Raf/ERK and MEK inhibitor treatments in beta cells (95, 180, 217). Additionally, our finding that ERK is further activated in response to the amplifying signal is consistent with its potentiated activation by factors like GLP-1 (218, 219). Furthermore, inhibition of ERK activation was shown to prevent GLP-1-potentiated secretion in INS-1 cells, implicating it further in the amplifying pathway (217). Combined, my data contrast with earlier findings that ERK was involved only in a mitogenic role in beta cells (220). It is possible that use of the older MEK inhibitor, PD98059, may have contributed to the controversy. There were no defects in insulin content in islets treated with PD03 for short periods (2 h), suggesting that expression of insulin at the protein level was not impacted by the blocked ERK activation. It is unclear in cases where ERK signaling has been blocked for short periods whether transcriptional effects might account for reduced secretory capacity. Future investigations will require large-

scale gene expression analyses of basal and glucose-stimulated beta cells under time courses with MEK inhibitors like PD03 to determine whether changes in gene expression may underlie any of ERK's roles in GSIS. Our results in PD03-treated mouse islets do support published work showing ERK has a role in secretion from perfused islets (180), although we found the effect of ERK activation to consistently be required for a full response to a subsequent stimulation with KCl as opposed to effects in first or second phase GSIS. Prior work has shown that inhibition of MEK with PD98059 in rat islets did not impair Ca^{2+} uptake (180) and we saw no defect in the first phase of glucose stimulated insulin secretion, suggesting that the PD03-induced defect in secretion after the rest period was not due defective Ca^{2+} influx. Differences between mouse and rat islets or potential off-target effects of PD98059 could underlie why perfusion of rat islets with PD98059 caused an overall reduction in basal and biphasic insulin secretion (180), while in our study perfusion of mouse islets with PD03 only blunted the response to a subsequent stimulation. Because PD03 likely blocked ERK activation in other cell types of the islet, potentially confounding our results by indirectly affecting the islet beta cells, we chose to investigate the effects of PD03 treatment on insulin secretion using MIN6 cells which contains only beta cells. Our studies in MIN6 cells also showed that ERK is necessary for cortical F-actin remodeling. Although prior studies suggested that inhibition of ERK signaling had no impact on glucose-induced F-actin remodeling, those prior studies focused on stress fibers, not cortical F-actin (96). Since ERK has a partial role in amplification, this suggests that signaling pathways in addition to ERK emanating from PAK1 may fulfill the remainder of the role of PAK1 in amplification. Studies assessing other PAK1 targets in beta cells and their potential roles in F-actin remodeling and insulin secretion are currently ongoing in our lab.

Since PAK1 is required for second phase insulin secretion, the question arises as to whether PAK1 signaling cross-talks with other pathways such as the GLP-1 stimulated signaling pathway to impact second phase GSIS. If PAK1 is activated downstream of cAMP, this could suggest integration with GLP-1 signaling to enhance second phase insulin secretion. GLP-1 acts

on the beta cell via the GLP-1 receptor which activates adenylyl cyclase to produce cAMP which activates both PKA and Epac2 (221). GLP-1 is one of the only known therapeutics that can increase both phases of insulin secretion in diabetic patients (222). Since signaling through both cAMP-PKA and PAK1 act in second phase insulin secretion it is tempting to speculate that PAK1 may have a role in GLP-1 action. PKA activation is also important for ERK activation. Multiple stimuli (GLP-1, forskolin) that increase cAMP levels in beta cells also potentiate ERK activation (219, 223). Consistently, PKA inhibitors (H-89) prevent glucose stimulated ERK activation (180, 219). Recent work suggests that cAMP might signal to PAK1 activation in beta cells through the kinase called Synapses of Amphids Defective (SAD-A) (224), although there are conflicting roles for SAD-A in insulin secretion which require further investigation (225). SAD-A can directly activate PAK1 (224), although it is unknown whether this depends on Cdc42. Therefore, future studies will be required to fully determine the linkage between Cdc42-PAK1, cAMP-PKA, and ERK signaling in glucose-stimulated biphasic insulin secretion and F-actin remodeling.

While PAK1 is required for downstream activation of Rac1 and ERK in beta cells, the identities of direct substrates for PAK1 in the beta cell are lacking. PAK1 has many known targets in other cell types that are likely beta cell targets as well, including MEK, Filamin A, MLC2, and RhoGDI. As a start, I have found that blocking PAK1 activation reduced MEK activation. This opens the possibility that MEK may be a target of PAK1 in response to glucose, although PAK1 could be signaling through Raf to MEK. Secondly, I have observed Filamin A and MLC2 phosphorylation in response to glucose; although it is currently unknown whether these events depend on PAK1 (Kalwat and Thurmond, unpublished data). Third, RhoGDI phosphorylation by PAK1 is known to cause its dissociation from Rac1 (84), and phosphomimetic mutants of RhoGDI show decreased Rac1 association in beta cells (44). These targets, and potentially others, may account for PAK1's effects on insulin secretion that cannot be attributed to ERK alone.

F-actin cytoskeletal architecture seems to have a role in regulating amplitude of glucose-stimulated ERK activation (96). Depolymerization of F-actin with latrunculin led to potentiated glucose-stimulated ERK activation (96). This active ERK was shown to be localized to the tips of actin filaments (96), placing it in the proper compartment to 1) act on proteins that could be involved in insulin secretion and 2) to be regulated downstream of PAK1. However, other well-characterized candidate PAK1 target pathways that might have a role in glucose-induced F-actin remodeling such as LIM kinase-cofilin, PAK1-Filamin A, and MLCK-myosin regulatory light chain 2 (MLC2) must also be considered. With regards to LIMK-cofilin, we recently demonstrated that PAK1 KO islets were unresponsive to changes of phospho-cofilin/cofilin under any conditions (45). Moreover, no changes in phospho-cofilin/cofilin content were observed in MIN6 beta cells, suggesting against LIMK-cofilin as a primary route for PAK1 signaling to the actin cytoskeleton (45). Filamin A is a known target of PAK1, specifically Filamin A Ser 2152, and phosphorylation at this site is necessary for Filamin's role in F-actin dynamics (90, 226). Our preliminary observations suggest Filamin A Ser 2152 is phosphorylated in beta cells in response to glucose (data not shown), but this will require future investigation for importance as a PAK1 target in the beta cell. PAK1 also targets MLCK and MLC2 phosphorylation by MLCK is known to promote actin remodeling processes in other cell systems (166). Phosphorylated MLC has been reported in MIN6 beta cells (227) with MLC2 phosphorylation of serine19 induced in response to glucose stimulation (169, 228). Since ERK can signal to MLCK in other cell types, it remains possible that PAK1 signals through MLCK/MLC2 directly and/or indirectly via ERK. Experiments are underway to determine the relative importance of PAK1 signaling to MLCK-MLC2 for glucose-induced actin remodeling.

Our results showed that inhibition of PAK1 with IPA3 led to a decrease in Syntaxin 4-F-actin binding under unstimulated conditions, as well as loss of glucose-regulation of this complex. Disruption of Syntaxin 4-F-actin complexes can have positive or negative effects on insulin secretion or insulin granule recruitment to the PM depending on the circumstances. For

example, under normal conditions glucose stimulation dissociates Syntaxin 4-F-actin complexes and is correlated with normal GSIS (33, 64, 143). Addition of a small competitive peptide known to disrupt Syntaxin 4-F-actin complexes results in potentiated GSIS and VAMP2-bound insulin granule accumulation at the PM (64). Similarly, latrunculin treatment depolymerizes F-actin and thereby releases Syntaxin 4 from F-actin association, which also correlates with potentiated GSIS (33, 64). Conversely, disruption of Syntaxin 4-F-actin as well as Syntaxin 4-Gelsolin complexes using a different competitive peptide leads to increased basal insulin release from mouse islets (143). Syntaxin 4 leads to increased basal secretion as well (210). In each of these three cases, increased basal or glucose-stimulated insulin secretion is associated with increased Syntaxin 4 accessibility to docking VAMP2-bound insulin granules. As such, it will be imperative in future studies to detail downstream PAK1 signaling to ERK and other substrates to understand how PAK1 regulates F-actin remodeling and Syntaxin 4-F-actin complex dynamics as they pertain to GSIS.

In summary, I determined that PAK1 is required for glucose-stimulated F-actin remodeling and VAMP2 accumulation in plasma membrane fractions. Additionally, I showed that PAK1 signals to the ERK pathway through MEK in beta cells and that MEK-ERK signaling is necessary for normal glucose-induced insulin secretion and F-actin remodeling. Finally, I linked PAK1 to Syntaxin 4-F-actin complex regulation, implicating the Cdc42-PAK1 pathway in the final steps of exocytosis. Since PAK1 has been implicated in both glucose-stimulated insulin secretion and insulin-stimulated glucose uptake, it is clear that specifically enhancing PAK1 signaling in certain tissues is a potential strategy for therapeutic intervention for type 2 diabetes. Since PAK1 protein levels are significantly decreased in type 2 diabetic human islets, treatments that act through the Cdc42-PAK1 pathway may not be effective unless PAK1 levels are restored. In addition, these results have implications in other secretory cell systems relevant to type 2 diabetes where PAK1 may play a vital role, such as GLP-1 secretion from enteroendocrine L cells of the gut (229).

Acknowledgments

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CHAPTER 3. GELSOLIN ASSOCIATES WITH THE N-TERMINUS OF SYNTAXIN 4 TO REGULATE INSULIN GRANULE EXOCYTOSIS

Some of the text in this Chapter is reproduced from:

Kalwat MA, Wiseman DA, Luo W, Wang Z, and Thurmond DC. Gelsolin Associates with the N Terminus of Syntaxin 4 to Regulate Insulin Granule Exocytosis. *Mol Endocrinol.* 2012. Jan;26(1):128-141.

Author Contributions: Wiseman DA generated data for figure 3-8A. Luo W generated data for figure 3-1B. Wang Z assisted with phospho-PAK1 blotting in figure 3-8C. The rest of the figures and text were generated by Kalwat MA.

3.1 INTRODUCTION

In response to a sharp increase in glucose concentration such as that induced by intake of a meal, pancreatic islet beta cells secrete insulin, doing so in a highly regulated and biphasic manner (30). The first phase is rapid and robust, occurring within 10 minutes of stimulation, and thought to be accounted for by a readily-releasable pool of granules already present at the PM. The second phase follows at a sustained, lower rate of secretion that can last for hours (32), and is elicited only in response to fuel-type secretagogues such as glucose. In order for insulin to be secreted, insulin granules must fuse with the PM in a stimulus-dependent and highly regulated manner; this process is carried out by soluble N-ethylmaleimide sensitive factor attachment receptor (SNARE) proteins (214, 230-232). Two known functional target membrane-SNARE (t-SNARE) isoforms from the Syntaxin protein family, Syntaxin 1A and Syntaxin 4, facilitate selective insulin granule fusion in beta cells via functioning as docking sites at the PM; Syntaxin 1A is required only for first phase secretion, while Syntaxin 4 is important for both first and second phases (39, 42). Both syntaxin isoforms pair with a second t-SNARE, SNAP-25, under both unstimulated/basal and stimulated conditions (33, 64). Insulin granules are equipped with the vesicle-SNARE (v-SNARE) protein VAMP2/synaptobrevin (194), and upon arrival at the PM, form heterotrimeric SNARE core complexes by docking with t-SNAREs to facilitate membrane fusion (reviewed in (192, 195)). Importantly, insulin release must be clamped under basal conditions, to provide the steep gradient necessary for a regulated stimulus response to appropriate secretagogues. Defects in clamping unsolicited insulin exocytosis are associated with elevated basal insulin levels and are disruptive to maintenance of glucose homeostasis *in vivo* (233).

To sustain insulin release, mature insulin granules in intracellular storage pools must be mobilized toward the PM. This process appears to coincide with glucose-induced remodeling of the actin cytoskeleton (33, 65, 128). F-actin functions as a barrier to restrain insulin granule

accumulation at the PM (1, 109), such that its depolymerization results in more morphologically docked granules and may confer release competence (34, 64). Evidence of positive effects of the cytoskeleton in stimulus-induced insulin secretion exists as well (129-132). In fact, insulin granules are known to interact with F-actin filaments, and require microtubule tracks upon which to traffic to the F-actin network toward the PM (133, 234, 235). Beyond impact upon trafficking, disruption of F-actin remodeling with agents that induce either F-actin disassembly or polymerization reveals that both actions jointly impact stimulated exocytosis outcomes (236). F-actin reorganization is important for stimulated exocytosis in many cell types other than beta cells, including neuroendocrine cells, adrenal chromaffin cells, platelets, and endothelial cells (119, 237, 238). In addition to controlling stimulated exocytosis, the actin cytoskeleton affects control of basal exocytosis through Focal Adhesion Kinase, EphA-Ephrin-A signaling, and cell contact (132, 136, 239). However, molecular mechanisms to describe how the cytoskeleton might participate in clamping of exocytotic machinery to ensure low levels of insulin secretion in the absence of stimuli remain unresolved.

Multiple SNARE proteins are linked with the F-actin cytoskeleton (64, 119, 205), although only Syntaxin 4 can directly interact with F-actin *in vitro*. Granule docking at Syntaxin 4 sites may be an important limiting factor in insulin exocytosis, given that increasing the number of Syntaxin 4 docking sites *in vivo* augments biphasic insulin secretion, while maintaining normal low basal levels (39). Syntaxin 4 binds to F-actin via a spectrin-like region within the first two coiled-coil domains of the N-terminus of Syntaxin 4 (64). Use of this region as a competitive peptide inhibitor reduces endogenous F-actin-Syntaxin 4 complex formation, concomitant with enhanced glucose-stimulated insulin secretion from MIN6 beta cells. While these data argue that F-actin anchors at Syntaxin 4 sites at the plasma membrane, the potential roles of multiple actin binding proteins which are implicated in, or shown to be necessary for, proper glucose-stimulated insulin secretion have yet to be tested for integration into this particular mechanism (35, 43, 96, 129). One particular protein of interest is Gelsolin, an F-actin-severing/capping protein that plays

a positive role in insulin secretion, and has been proposed to be important for glucose-induced F-actin remodeling which occurs in beta cells through an undefined mechanism (96, 153).

In this study, we provide the first evidence for formation of a novel and direct interaction between Syntaxin 4 and Gelsolin, mediated via the N-terminal Ha domain of Syntaxin 4. In beta cells, this complex is dissociated in response to acute glucose- or KCl-stimulation. Introduction of an Ha-domain peptide (amino acid residues 39-70) into MIN6 beta cells mimicked the action of these secretagogues by inducing dissociation of endogenous Syntaxin 4-Gelsolin complexes. Functionally, these binding alterations were accompanied by elevated basal insulin release in mouse islet and MIN6 beta cells, coordinate with inappropriate activation of Syntaxin 4. The peptide-induced dissociation of Syntaxin 4-Gelsolin complexes in MIN6 beta cells also attenuated acute glucose- and KCl-stimulated insulin exocytosis, coinciding with attenuation of the K_{ATP} -channel dependent triggering pathway in the diazoxide paradigm. These data support a new model for a clamping mechanism underlying regulated SNARE protein-mediated exocytosis. Given that 1) Gelsolin and Syntaxin 4 are ubiquitously expressed proteins; 2) F-actin remodeling occurs in non-beta cell types; and 3) the effects upon insulin exocytosis are not secretagogue-specific, we propose that this mechanism may be applicable to regulated exocytosis events on a broader cell biological basis.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Two rabbit polyclonal anti-Syntaxin 4 antibodies were obtained for use in coimmunoprecipitation (Chemicon, Temecula, CA) and for immunoblotting (in-house, described in (210)). Rabbit anti-GFP and anti-GAPDH antibodies were obtained from Abcam (Cambridge, MA). Monoclonal mouse anti-GFP, mouse anti-Cdc42, and mouse anti-Gelsolin antibodies were purchased from Clontech/BD Biosciences (Mountain View, CA). Rabbit anti-actin antibody,

Flag-M2 antibody, DAPI, nifedipine and diazoxide were purchased from Sigma (St. Louis, MO). Latrunculin B was obtained from Calbiochem. Rabbit anti-GST was obtained from Affinity Bioreagents (Golden, CO). Rabbit anti-phospho- α Pak1 (Thr423) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit IgG and rabbit anti- α Pak1 were purchased from Cell Signaling (Danvers, MA). Fura-2/AM and Rhodamine-Phalloidin were purchased from Invitrogen (Carlsbad, CA). The MIN6 cells were a gift from Dr. John Hutton (University of Colorado Health Sciences Center). Goat anti-mouse horseradish peroxidase secondary antibody was obtained from Thermo Fisher Scientific (Rockford, IL). Goat anti-rabbit horseradish peroxidase secondary antibody and TransFectin lipid reagent were acquired from Bio-Rad (Hercules, CA). ECL reagent and Supersignal Femto were purchased from GE Healthcare (Piscataway, NJ) and Pierce, respectively. The rat insulin radioimmunoassay kits were obtained from Millipore (Billerica, MA).

3.2.2 Plasmids

The pGEX4T-1 plasmids containing rat Syntaxin 4 residues 1-273, -1-194, -1-112, and 1-70 and pEGFP-C2 plasmids containing Syntaxin 4 residues 1-273, 1-194, and 39-112 were previously described (64). A PCR-generated DNA insert comprising the 39-70 residue region of Syntaxin 4 was subcloned into the 5' EcoRI and 3' XhoI sites of the pEGFP-C2 vector (Clontech) to generate pEGFP-Syntaxin 4-39-70; GFP-Scr (scrambled helical peptide) was inserted similarly using annealed primer set sequences: 5'sense strand:

attcatgaagttcttcgacaccaagaaggtggagctggagctgaccagatcgtgcagatggagcagatcgagcagctgagcaggaagcagaccagggccgtgtagg-3'. GFP-Scr helicity was predicted to be 87.5%, that closest in helicity to GFP-39-70 that also failed to match with any known mouse peptides in BLAST. The pGFP-39-70-Ad adenoviral expression plasmid was generated by subcloning the GFP-39-70 DNA into the 5' BamHI and 3' NotI sites of the pAdCMVK vector (Viraquest, Inc., North Liberty, IA); after which adenoviral production was carried out by Viraquest, Inc. The pIRES-GFP-Gelsolin-Flag

plasmid was generated by subcloning DNA comprising only the cytoplasmic isoform (missing residues 1-50 of full-length protein) from the full length mouse cDNA purchased from Openbiosystems (Huntsville, AL) into the 5' NheI and 3' XhoI sites of the pIRES-GFP-3XFlag vector; the modified vector was a gift of Dr. Raj Khanna (Indiana University School of Medicine, IN). All constructs were verified by DNA sequencing.

3.2.3 Recombinant Proteins and Interaction Assays

All GST fusion proteins were expressed in *Escherichia coli* and purified by glutathione-sepharose affinity chromatography as described previously (64). GST-Syntaxin 4 truncation GST proteins (20 µg) immobilized on sepharose beads were incubated with 20 µg of recombinant his-tagged human Gelsolin protein (Cytoskeleton, Inc) in a 0.25 % NP-40 containing lysis buffer (25 mM HEPES, pH 7.4, 0.25% Nonidet P-40, 10% glycerol, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 137 mM NaCl, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 1 µg/ml pepstatin, 5 µg/ml leupeptin) for 2 h at 4°C. Following three washes with PBS, bound proteins were eluted from the sepharose beads and proteins resolved on 10% SDS-PAGE followed by transfer to PVDF membrane for immunoblotting.

3.2.4 Cell Culture, Transient Transfection, Adenoviral Transduction, and Secretion Assays

MIN6 beta cells were cultured in DMEM (25 mM glucose) supplemented with 15% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, 292 µg/ml L-glutamine, and 50 µM β-mercaptoethanol as described previously (201). MIN6 beta cells at ~60-70% confluence were transfected using cesium chloride-purified plasmid DNA with TransFectin (Bio-Rad) to obtain ~30-50% transfection efficiency. Electroporation of MIN6 cells was carried out as previously described (240). After 48 h of incubation, cells were washed twice with and incubated for 2 h in freshly prepared modified Krebs-Ringer bicarbonate buffer (MKRBB: 5 mM KCl, 120

mM NaCl, 15 mM HEPES, pH 7.4, 24 mM NaHCO₃, 1 mM MgCl₂, 2 mM CaCl₂, and 1 mg/ml radioimmunoassay-grade BSA). Cells were stimulated with 20 mM glucose for the times indicated in the figures, after which the buffer was collected and centrifuged for 10 min at 4°C to pellet cell debris, and insulin secreted into the buffer was quantitated using a rat insulin radioimmunoassay kit (Millipore). Cells were harvested in 1% NP-40 lysis buffer (same as 0.25% NP-40 with exception of 1% Nonidet-P40), lysed for 10 min at 4°C, and were cleared of insoluble material by centrifugation for 10 min at 4°C for subsequent use in co-immunoprecipitation experiments. For adenoviral overexpression, MIN6 cells at 50-60% confluence were transduced at an MOI of 100 for 2 h, washed twice with PBS, and incubated in complete media for 48 h. Cells were subsequently preincubated in MKRBB, subjected to stimulation, and harvested for generation of detergent cell lysates as described above.

3.2.5 Co-immunoprecipitation and Immunoblotting

For each immunoprecipitation, 2-3 mg of cleared detergent lysate protein was combined with 1 µg of antibody per mg protein and the reaction rotated for 2 h at 4°C. Protein G Plus agarose beads (Santa Cruz) were added and reactions rotated at 4°C for an additional 2 h. Beads were pelleted and washed three times with lysis buffer and resulting immunoprecipitates were resolved on 10-12% SDS-PAGE and transfer to PVDF membranes for immunoblotting. Immunoreactive bands were visualized with ECL or Supersignal Femto reagents and imaged using a Chemi-Doc gel documentation system (Bio-Rad).

3.2.6 Calcium Imaging

MIN6 cells transduced as described above were preincubated in MKRBB for 2 h, with Fura-2 AM (5 µM) added to the cells for an additional 25 min as reported previously (211, 241). Cells were washed with MKRBB to remove excess Fura-2 AM and placed in fresh MKRBB containing low (2 mM) glucose and KCl (5 mM). Cells were imaged under constant perfusion (1

ml/min) for 300 seconds, followed by stimulation with 35 mM KCl to elicit calcium influx for 300 seconds. Fura-2 AM was excited at 340 and 380 nm, and emission captured at 510 nm on a Zeiss Axio Observer Apochromat 100X/1.46 objective equipped with a Hamamatsu Orca-ER digital camera and analyzed using AxioVision 4.7 software (Carl Zeiss, Germany). Fura-2 has been shown to exhibit similar fluorescence from cells untransfected or transfected with GFP (242).

3.2.7 Immunofluorescence and Confocal Microscopy

MIN6 cells plated onto glass coverslips at 30% confluence were transiently transfected with 4 µg of plasmid DNA/35 mm well. After 48 h incubation, cells were placed in MKRBB for 2 h, followed by stimulation with 20 mM glucose for 5 min and then immediately fixed and permeabilized in 4% paraformaldehyde and 0.1% Triton X-100 for 10 min at 4°C. Fixed and permeabilized cells were blocked in 1% BSA plus 5% donkey serum for 1 h at room temperature, followed by incubation with 0.17 µM Rhodamine-Phalloidin for 1 h, per manufacturer instructions. MIN6 cells were then washed three times with phosphate-buffered saline (pH 7.4). During the final wash, DAPI was added to stain nuclei. All cells were washed again with PBS, and mounted (using Vectashield) for confocal fluorescence microscopy. EGFP and Rhodamine fluorescing cells were imaged using single-channel scanning with a ×60 objective under a 2X zoom using an Olympus FV1000-MPE confocal microscope (Olympus, Center Valley, PA).

3.2.8 Mouse Islet Isolation, Transduction, Perfusion, and Static Culture

All studies involving mice followed the Guidelines for the Use and Care of Laboratory Animals at Indiana University School of Medicine. Wild type male C57BL/6J mice were sacrificed for pancreatic islet isolation as previously described (39). All viruses were obtained from Viraquest, Inc. Freshly isolated islets were immediately transduced with 10^7 pfu/islet with either GFP-Ad or GFP-39-70-Ad CsCl-purified particles for 1 h at 37°C in RPMI-1640 medium,

washed twice with PBS and incubated 48 h at 37°C/5% CO₂. GFP fluorescence was visualized in greater than 60-70% of islet cells with even penetration to the islet core. GFP positive islets were handpicked into batches of 10/tube for static secretion studies or 50 islets/column for perfusion. Perfusion studies were carried out as previously described (200). For static culture, islet batches were incubated 2 h in KRBH buffer (10 mM HEPES pH 7.4, 134 mM NaCl, 5 mM NaHCO₃, 4.8 mM KCl, 1 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄ containing 0.5 mg/ml BSA) supplemented with 2.8 mM glucose, followed by 1 h incubation in either 2.8 mM or 16.7 mM glucose. Insulin secreted into the buffer and insulin content in the corresponding islet lysates was quantified by Rat Insulin RIA (Millipore).

3.2.9 Statistical Analysis

All quantitated data are expressed as mean \pm S.E. Data were evaluated using Student's t test and considered significant if $p < 0.05$.

3.3 RESULTS

3.3.1 Syntaxin 4 directly interacts with Gelsolin

We first identified Gelsolin as a potential Syntaxin 4-binding partner in an unbiased yeast two-hybrid screen (D.C. Thurmond and J.E. Pessin, unpublished results), and subsequently validated Gelsolin protein to bind directly to recombinant soluble (lacking the C-terminal transmembrane domain) GST-Syntaxin 4 *in vitro* (Figure 3-1A). Since both Syntaxin 4 and Gelsolin are required for glucose-stimulated insulin exocytosis (39, 96), the presence of full-length endogenous Syntaxin 4-Gelsolin complexes was subsequently investigated, and revealed to exist in MIN6 beta cell lysates (Figure 3-1B). Notably, these complexes were found to rapidly and transiently dissociate in response to acute 5 min glucose stimulation. Following 30 min glucose stimulation, Syntaxin 4-Gelsolin complex abundance resumed to ~50% that detected

under basal conditions. Changes in complex formation were independent of changes in Syntaxin 4 or Gelsolin protein abundances in cell lysates.

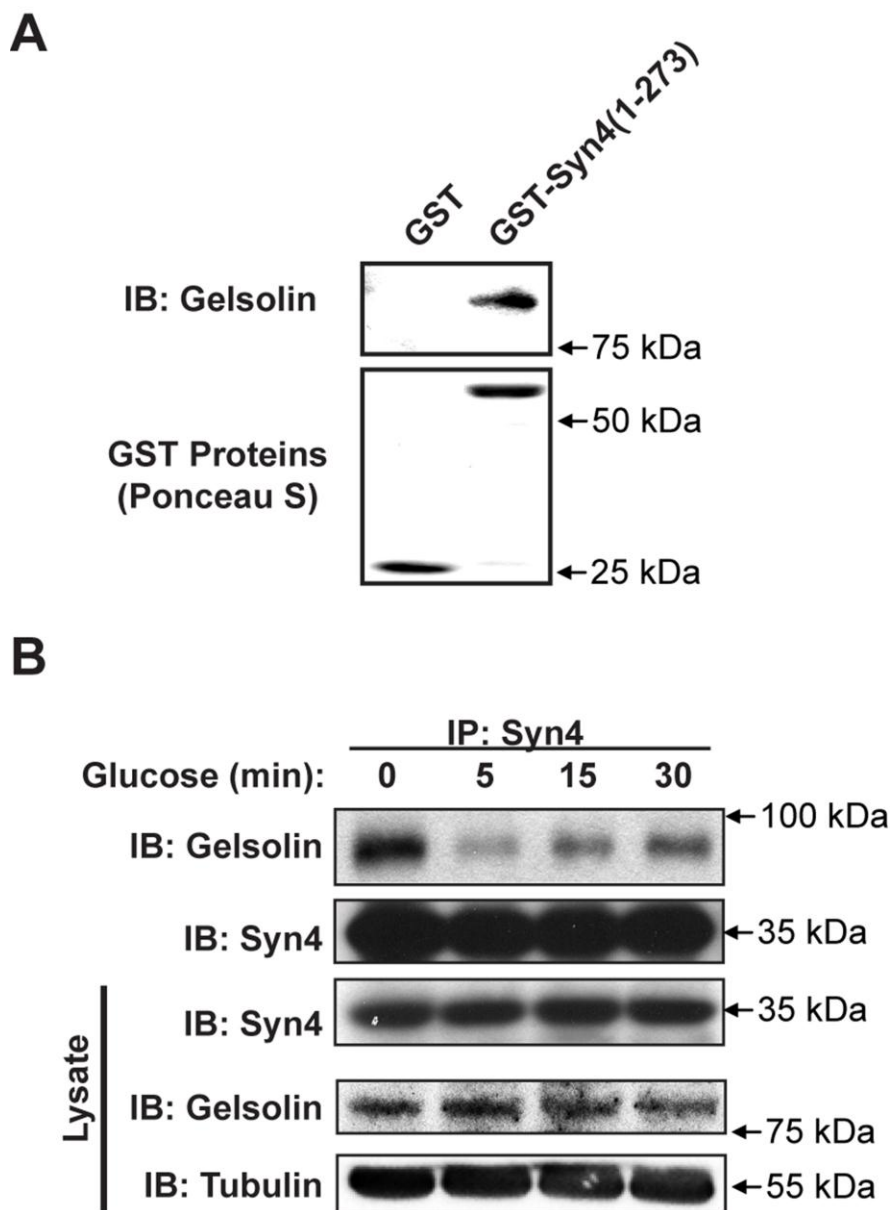


Figure 3-1. Syntaxin 4 and Gelsolin directly interact and form complexes in MIN6 beta cells that are sensitive to glucose stimulation. **A)** Recombinant Gelsolin was combined with either GST or GST-Syn4 (1-273) linked to sepharose beads for 2 h incubation at 4°C. Beads were pelleted and extensively washed, with eluates subjected to 10% SDS-PAGE for immunoblot detection (IB) of Gelsolin. Ponceau staining served as control for GST protein loading. Data are representative of at least three independent experiments using two different batches of protein. **B)** MIN6 cells were pre-incubated in MKRBB for 2 h and stimulated with 20 mM glucose for 5, 15 or 30 min. Cleared detergent cell lysates were prepared and used in Syn4 immunoprecipitation (IP) reactions. Immunoprecipitates were resolved on 10% SDS-PAGE and proteins were transferred to PVDF for immunoblot detection (IB) of Syn4 and Gelsolin. Protein abundances in starting input lysates (100 µg) were confirmed on a separate gel. Data are representative of at least three independent co-immunoprecipitation studies. Syn4, Syntaxin 4.

Given the novelty of this interaction, we next sought to identify the minimal region of Syntaxin 4 required to confer direct binding to Gelsolin. Syntaxin 4 truncations fused C-terminally to GST were analyzed using *in vitro* binding studies with recombinant Gelsolin. As depicted in the schematic of Figure 3-2A, Syntaxin 4 protein is composed of three N-terminal alpha-helical domains which, based upon comparison with the X-ray crystal structure of family member Syntaxin 1A, pack together to comprise a coiled-coil bundle, and are linked to the C-terminal alpha-helical SNARE domain known to be operational in binding SNARE proteins (202). Elimination of all but the N-terminal most Ha (residues 1-70) domain failed to ablate binding to Gelsolin, including the Hb (residues 71-112) region previously shown to be required to confer Syntaxin 4 binding to F-actin (64) (Figure 3-2B). To evaluate this binding event in a mammalian cell expression system, CHO-K1 cells were electroporated to express Flag-tagged Gelsolin with truncated GFP-Syntaxin 4 fusion proteins (GFP-39-112 and GFP-1-70), and detergent solubilized cell lysates were prepared for co-immunoprecipitation analyses. Indeed, anti-Flag (Gelsolin) immunoprecipitation co-precipitated with both truncated forms of Syntaxin 4 (Figure 3-2C). Following this, the far N-terminal residues 1-38 were removed to isolate the Ha domain, GFP-39-70, which was sufficient to confer binding to Flag-tagged Gelsolin (Figure 3-2D). Despite efforts to test for necessity of the Ha domain, deletion of the Ha from either GST- or GFP-fusion protein rendered proteins too unstable to study. Regardless, the ability of this Ha domain to confer binding to Gelsolin distinguishes its interaction with Syntaxin 4 from that of F-actin, since Ha alone failed to confer Syntaxin 4 binding to F-actin (64). Importantly, this is the first demonstration of the Ha domain conferring stable binding with any of the known Syntaxin 4 binding partners.

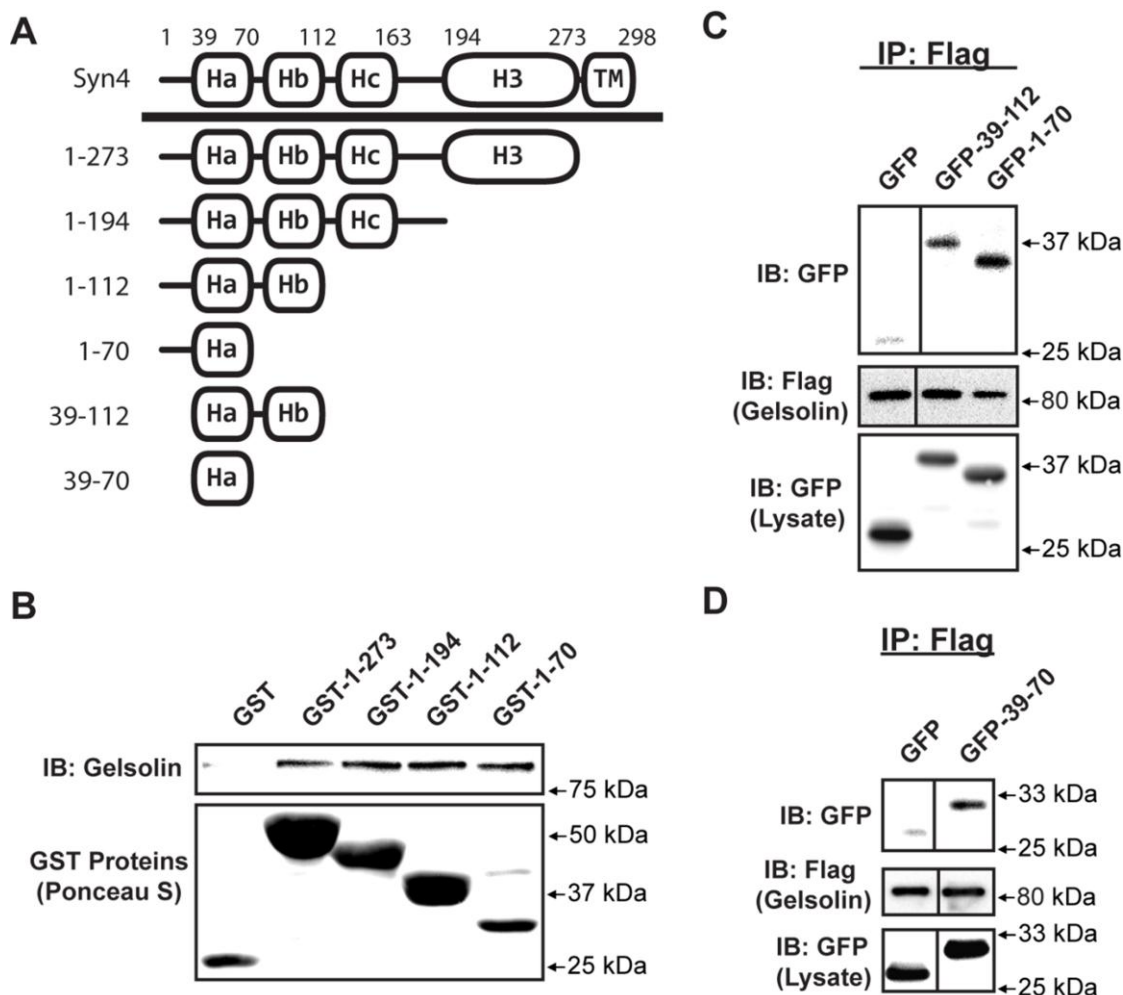


Figure 3-2. Residues 39-70 of Syntaxin 4 are sufficient to confer Syntaxin 4-Gelsolin binding. **A)** Depiction of the multiple helical domains of the Syn4 protein. The N-terminal helical domains are denoted as Ha, Hb, and Hc; the C-terminal H3 helix is the SNARE domain, followed by the transmembrane domain (TM). **B)** *In vitro* binding reactions containing recombinant Gelsolin protein plus either GST or GST-Syn4 truncation proteins linked to beads were performed as described in Figure 3-1, and proteins resolved by 10% SDS-PAGE for immunoblotting (IB). Ponceau S staining shows the presence of the GST proteins in each reaction. Data are representative of two independent experiments. **C)** CHO-K1 cells were co-electroporated with pEGFP or pEGFP-Syn4(39-112) and -(1-70) or **D)** pEGFP-Syn4(39-70) plasmids along with pIRES-Gelsolin-Flag DNA, and 48 h later detergent lysates were prepared for immunoprecipitation (IP) with anti-Flag antibody. Immunoprecipitates were subjected to 10% SDS-PAGE for immunoblot detection (IB) of GFP and Flag (Gelsolin). Input lysates (50 μ g) demonstrate expression and migration of each protein. Vertical lines denote splicing of lanes from within the same gel. Data are representative of three independent immunoprecipitation experiments.

3.3.2 Competitive inhibition of endogenous Syntaxin 4-Gelsolin complexes

Since there are reported effects of apoptosis and compensatory cytoskeletal effects associated with Gelsolin knockdown/depletion approaches (152, 243), we opted to use the Ha-domain peptide (GFP-39-70) as a competitive inhibitor in effort to selectively target the Syntaxin 4-Gelsolin complex to evaluate its function in insulin exocytosis. In addition to the unusual ability of the Syntaxin 4 Ha domain to confer binding to Gelsolin, as opposed to how Syntaxin 4 binds other partners, the Ha domain (residues 39-70) carries the least identity of all its helical domains (< 35%) to Syntaxin 1A. Using lysates prepared from MIN6 beta cells expressing the GFP-39-70 protein, anti-Syntaxin 4 immunoprecipitation reactions resulted in attenuated co-precipitation of Gelsolin under basal (unstimulated) conditions, compared to reactions from control GFP; a scrambled (GFP-Scr) peptide, designed to retain helical structure (<http://npsa-pbil.ibcp.fr>), was without effect (Figure 3-3A). Having established specificity of the GFP-39-70 peptide upon Syntaxin 4-Gelsolin complexes, we next evaluated effects following an acute 5 min glucose stimulation. GFP expressing cells showed reduced interaction of endogenous Syntaxin 4-Gelsolin complexes in GFP expressing cells, similar to that of complexes shown in Figure 3-1B, however glucose stimulation of GFP-39-70 expressing cells failed to further dissociate the endogenous complex (Figure 3-3B, i). As we have previously shown that F-actin, but not G-actin, interacts with Syntaxin 4 in beta cells in a glucose-sensitive manner, and can directly interact with Syntaxin 4 *in vitro* (64), we questioned whether Gelsolin was coupled to this interaction. Coordinate with this, F-actin association with Syntaxin 4 in the presence of the GFP-39-70 peptide was diminished in unstimulated cell lysates, relative to GFP alone (Figure 3-3B, ii). Moreover, pharmacologically-induced actin depolymerization using Latrunculin B (LAT) caused dissociation of both actin and Gelsolin from Syntaxin 4 (Figure 3-3C).

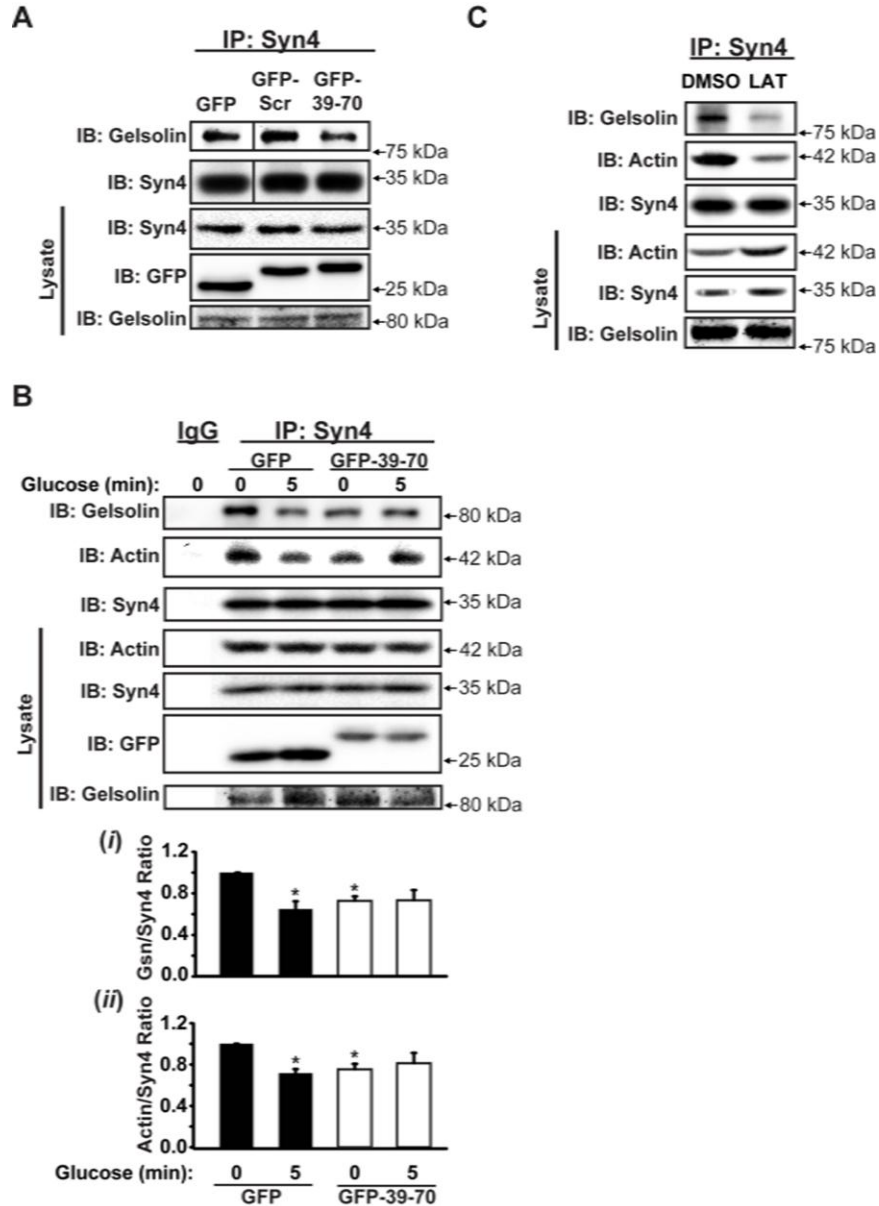


Figure 3-3. GFP-39-70 disrupts endogenous Syn4-Gelsolin complexes. **A)** MIN6 cells transfected to express GFP, GFP-39-70, or GFP-Scr (scrambled peptide) were preincubated in MKRBB for 2h and resultant lysates used for anti-Syn4 immunoprecipitation (IP). Coprecipitated proteins were resolved on 10% SDS-PAGE for immunoblot detection (IB). Data represent at least three independent co-immunoprecipitation experiments. **B)** MIN6 cells transfected to express either GFP or GFP-39-70 proteins were pre-incubated and stimulated with 20 mM glucose for 5 min; lysates were used in IP reactions as detailed in part A above. Band intensities of actin and Gelsolin association with Syn4 were quantitated and expressed as the ratio of Gelsolin (Gsn) (i) and actin (ii) to Syn4 (normalized, basal ratio = 1 for each experiment). Bars represent the mean \pm S.E. of the three independent experiments (* $p < 0.05$, versus unstimulated GFP). **C)** MIN6 cells were treated with vehicle (DMSO) or 10 μ M latrunculin B (LAT) for 2 h in MKRBB and resultant cell lysates used for IP as described in parts A and B above in three independent experiments. Protein abundances in starting input lysates (100 μ g) were confirmed on a separate gel. Vertical lines denote splicing of lanes from within the same gel.

Since Gelsolin knockdown in MIN6B1 cells exerted global effects upon F-actin remodeling, we investigated this as a potential explanation for the GFP-39-70-induced alterations to Syntaxin 4 binding. We first determined that selective attenuation of Syntaxin 4-Gelsolin binding under basal conditions was not attributable to differential GFP-39-70 subcellular localization, as its principally cytosolic localization was similar to that of GFP, as visualized by confocal microscopy (Figure 3-4, panels 1-4). However, unstimulated GFP-39-70 expressing cells contained contiguous cortical F-actin (red, rhodamine-phalloidin staining) similar to that of neighboring untransfected cells and of GFP control cells (Figure 3-4, panels 5-6, 9-10). Moreover, GFP and GFP-39-70 expressing cells all showed the expected acute glucose-induced actin remodeling, as determined by disruption of the contiguous cortical F-actin rim encircling each cell (Figure 3-4, panels 7-8, 11-12). These data indicated that the GFP-39-70 peptide sufficed as a competitive inhibitor of endogenous Syntaxin 4-Gelsolin complexes present in unstimulated MIN6 beta cells, and did so without inducing global changes in cortical F-actin structure.

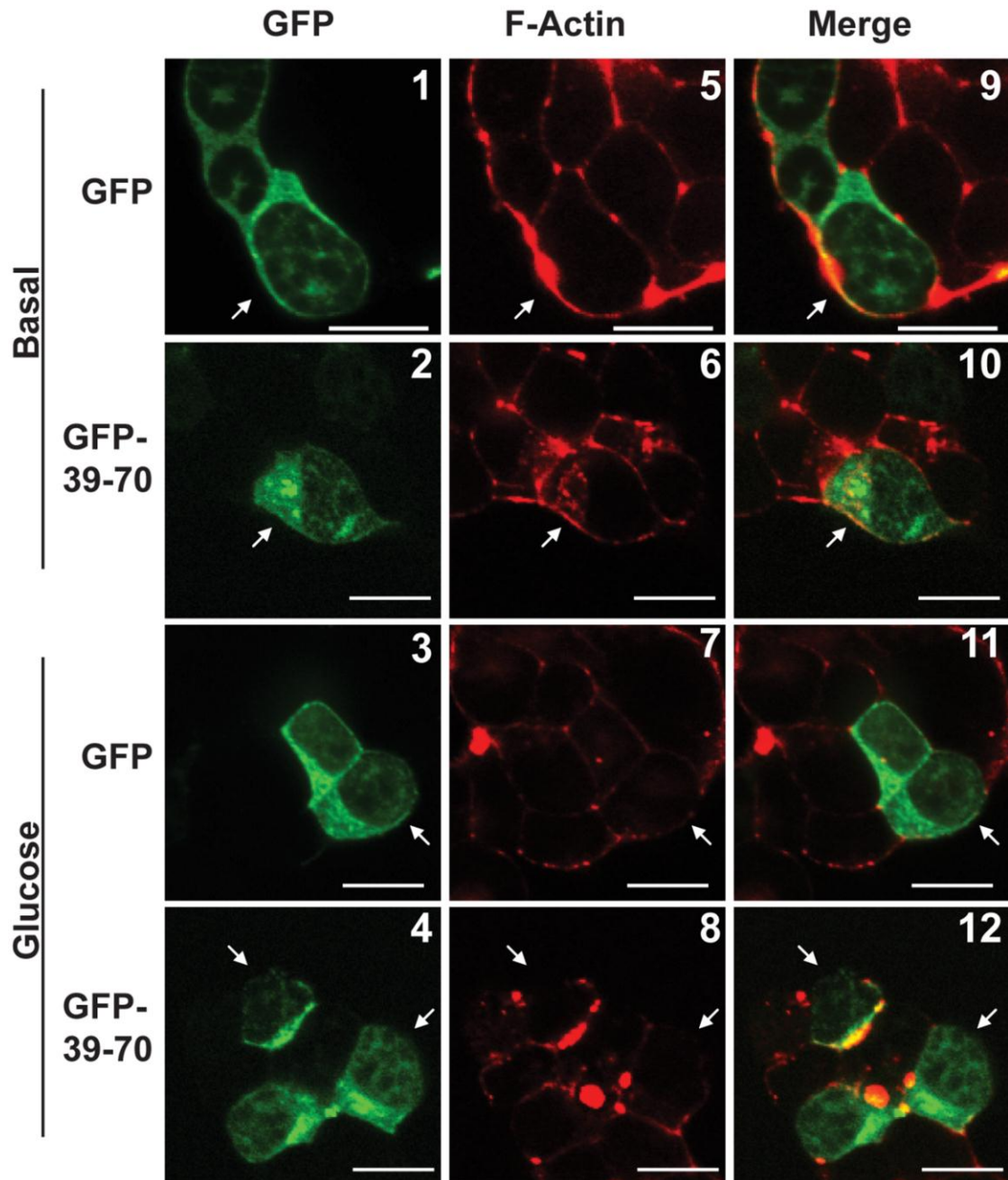


Figure 3-4. GFP-39-70 does not disrupt normal glucose-induced actin remodeling. MIN6 cells were plated on glass coverslips and transfected to express GFP or GFP-39-70 proteins (green) were pre-incubated in MKRBB for 2 h and left unstimulated (top panels 1-2, 5-6, 9-10) or stimulated with 20 mM glucose for 5 min (bottom panels 3-4, 7-8, 11-12). Cells were fixed, permeabilized and cortical F-actin staining detected by Rhodamine-Phalloidin staining (red). Cells in clusters containing both non- and GFP-transfected cells were imaged at the midplane of the cluster by single channel scanning confocal microscopy to determine subcellular distribution of the GFP proteins (panels 1-4) and F-actin remodeling (panels 5-8). Merged GFP and F-actin images are shown in panels 9-12 to permit comparisons between non-transfected and transfected cells. *Scale bar*, 10 μ m. Data shown are representative of three independent sets of experiments.

3.3.3 Syntaxin 4-Gelsolin complexes are required to clamp unsolicited insulin exocytosis events

The competitive peptide was subsequently used to determine the functional ramifications of disrupting Syntaxin 4-Gelsolin complexes upon insulin secretion from isolated islets and MIN6 cells. To ensure thorough and efficient expression of the peptide across cell populations for these studies, an adenovirus encoding GFP-39-70 (GFP-39-70-Ad) was generated and validated to exert dissociating actions upon endogenous Syntaxin 4-Gelsolin complexes akin to that of the plasmid-based delivery system (Figure 3-5). Subsequently, islets isolated from wild-type C57BL/6J mice were transduced with GFP-39-70 or GFP adenoviruses and protein expression confirmed (Figure 3-6A), albeit GFP-39-70 expression was consistently lower than that of GFP. Islet perfusion experiments were initiated to examine effects of GFP-39-70 expression upon basal and phasic insulin release. GFP-39-70 expressing islets released ~4-fold more insulin under basal (2.8 mM glucose) conditions (Figure 3-6B), and first phase secretion appeared to be slightly impaired in GFP-39-70 expressing islets (GFP-39-70 peaked 3-fold above basal, GFP peaked 10-fold above basal). However, defective basal secretion impeded reliability of area under the curve analysis to quantify phasic differences. We next employed a short term static incubation approach as a means to quantify acute/first phase secretion using glucose or KCl secretagogues. Again, basal elevation in GFP-39-70 expressing cells was fully recapitulated in the static culture system, yet short term stimulated secretions were highly erratic and thwarted efforts to discern significant first phase differences (Figure 3-6C). However, this rise in basal secretion in GFP-39-70 islets nearly abolished the overall stimulatory responses compared with those of GFP expressing islets (SI, stimulation index=glucose-stimulated/basal secretion) (Figure 3-6D). Differences in secretion were not due to alterations in insulin content (Figure 3-6E). No significant differences in long-term glucose-stimulated insulin secretion (1 h) or associated insulin content were noted between GFP and GFP-39-70 expressing islets (Figure 3-7A and B).

These data suggested that dissociation of endogenous Syntaxin 4-Gelsolin complexes under basal conditions permitted or otherwise prompted inappropriate insulin release.

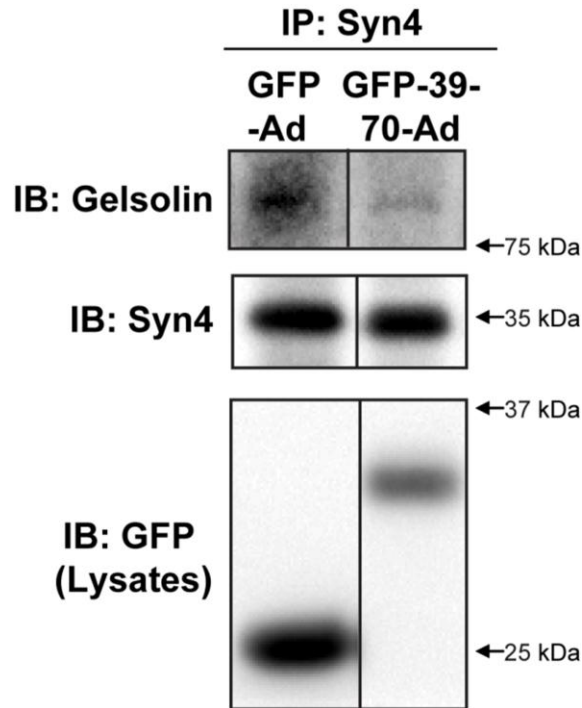


Figure 3-5. Adenoviral expression of GFP-39-70 results in disruption of endogenous Syn4-Gelsolin complexes in unstimulated beta cells. MIN6 cells were transduced to express either GFP or GFP-39-70, and following a 48 h incubation, were subjected to a 2 h preincubation in MKRBB and left unstimulated prior to harvest for preparation of cleared detergent lysates. Lysates were used in Syn4 immunoprecipitation (IP) reactions, and coprecipitated proteins were resolved on 10% SDS-PAGE for immunoblot detection (IB) of Syn4, Gelsolin, and GFP. Data are representative of two independent experiments.

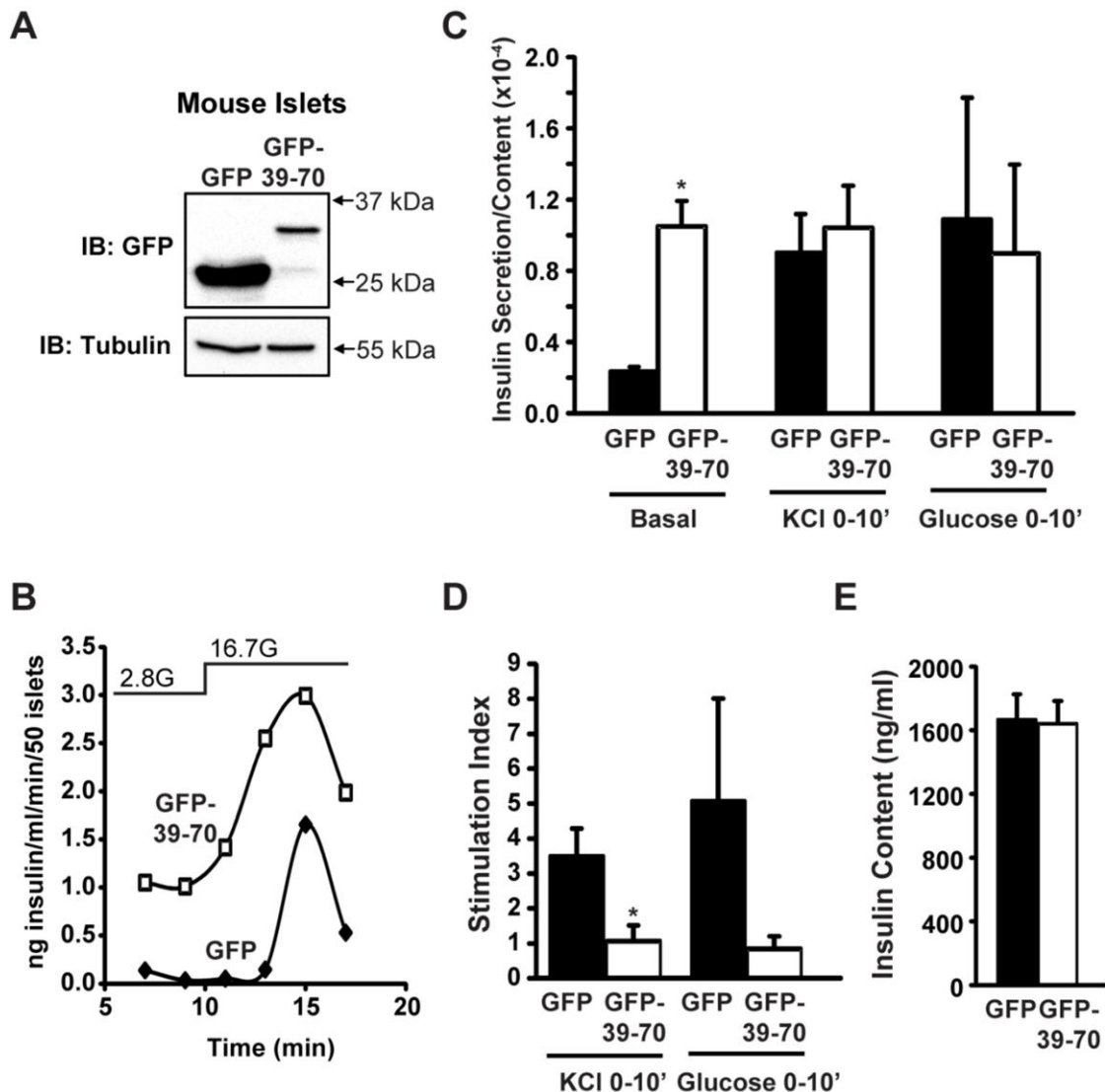


Figure 3-6. Disruption of Syn4-Gelsolin complexes elevates basal insulin secretion from isolated mouse islets. Freshly isolated mouse islets were immediately transduced with GFP or GFP-39-70 adenovirus, and following 48 h incubation GFP-positive islets were handpicked under a fluorescence microscope for immunoblot analysis (**A**), for perfusion analysis (**B**), and for static culture studies (**C**). Perfusion entailed use of 50 GFP positive islets per column with GFP and GFP-39-70 expressing islet columns run in parallel; islets were perfused with low glucose (2.8 mM) followed by high glucose (16.7 mM) to capture first phase release. Static secretion reactions contained 10 GFP positive islets/tube, and were incubated in low glucose (2.8 mM) KRBH for 2 h and buffer collected for quantitation of basal insulin secretion. Buffers containing stimulatory levels of glucose (16.7 mM) or KCl (35 mM) were added to the islets for 10 min, followed by collection for insulin quantitation. Insulin content in the remaining islets was also determined and used to normalize insulin secretion data; $*p < 0.001$, versus GFP. **D**) Stimulation index was calculated using the values from (C) ($SI = \text{stimulated insulin release/basal insulin release}$). **E**) Insulin content was similar between transduced islet groups. Data represent the mean \pm S.E. of three independent islet isolation experiments; $*p < 0.05$, versus GFP.

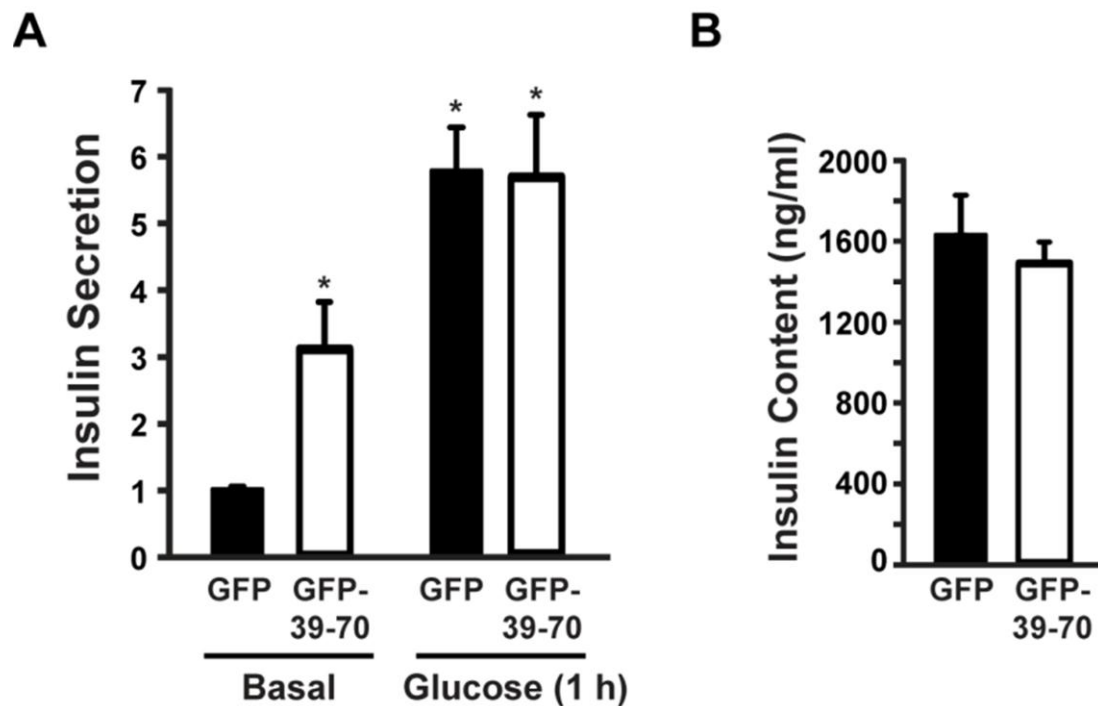


Figure 3-7. Adenoviral expression of GFP-39-70 increases basal insulin release from islet beta cells. **A)** Mouse islets transduced to express GFP or GFP-39-70 were assessed in static culture experiments for insulin release under basal (2.8 mM) or long-term glucose stimulated (20 mM for 1 h) conditions. Secretion data were normalized for insulin content in corresponding islets; **B)** insulin content was similar between transduced islet groups. Data represent the average \pm S.E. of three independent islet batches; * $p < 0.05$ versus unstimulated GFP islets.

Loss of clamping of basal insulin release could result from a direct effect upon Syntaxin 4 activation, or possibly an indirect effect of aberrant Cdc42 signaling, as has been found to occur in other cases of dysregulated basal insulin secretion (77, 210). To test for alterations in Syntaxin 4 activation, lysates prepared from MIN6 beta cells transduced to express GFP or GFP-39-70 protein were combined with recombinant GST-VAMP2 (soluble, TM domain deleted) protein, linked to sepharose beads, as a means to selectively precipitate open conformation Syntaxin 4 as described previously (64, 210). It is expected that opening increases accessibility of Syntaxin 4 to enhance granule docking to promote granule fusion and insulin release. While GFP expressing cells exhibited a low level of basal activation and responded to acute glucose stimulation (5 min) with the traditional ~2-fold increase in Syntaxin 4 activation, GFP-39-70 expressing cells showed significantly elevated basal Syntaxin 4 activation, with no further responsiveness to glucose (Figure 3-8A). These differences were not a consequence of differential Syntaxin 4 protein expression, or between GFP and GFP-39-70 in the starting lysates (Figure 3-8B). Further implicating Syntaxin 4 activation as a mechanism to explain elevated basal secretion, Cdc42 signaling was unaffected by the GFP-39-70 peptide, as gauged by normal activation (phosphorylation) levels of the immediate downstream Cdc42 effector protein PAK1 under basal conditions (Figure 3-8C).

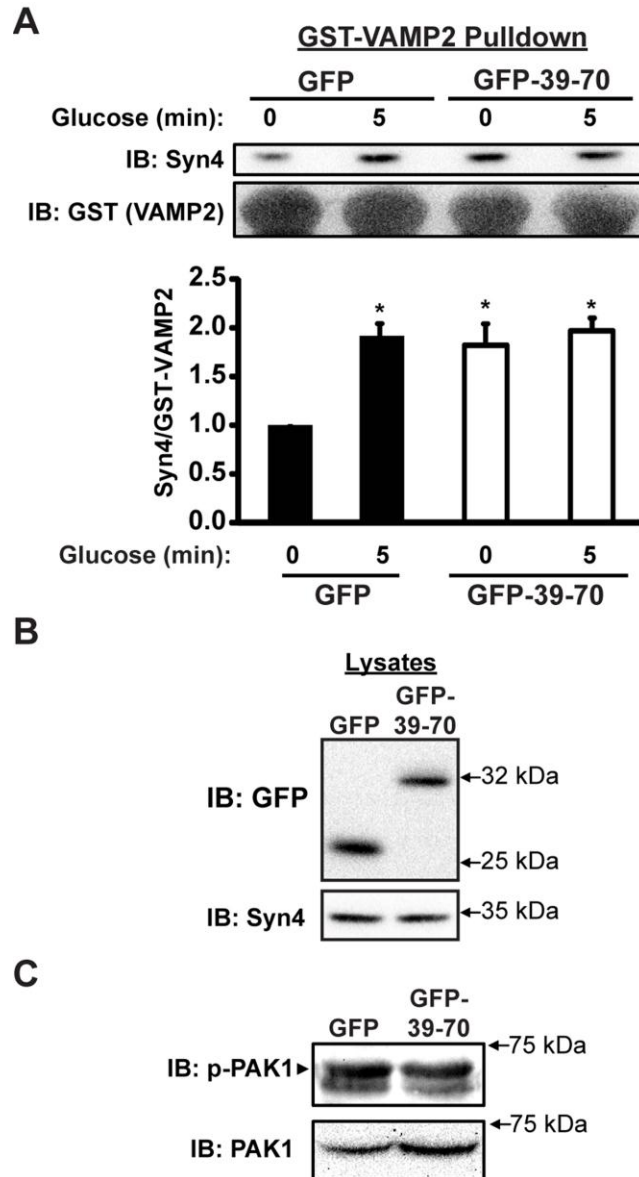


Figure 3-8. Disruption of Syn4-Gelsolin complexes triggers Syn4 activation in the absence of secretagogue stimulation. **A)** MIN6 cells transduced to express either GFP or GFP-39-70 proteins were pre-incubated in MKRBB for 2 h and left unstimulated or stimulated with 20 mM glucose for 5 min to generate cleared detergent cell lysates for use in GST-VAMP2 interaction assays. Lysate protein (2-3 mg) was combined with GST-VAMP2 (soluble, TM domain deleted) bound to glutathione sepharose beads, incubated for 2 h and precipitated proteins resolved on 10% SDS-PAGE for immunoblot detection (IB) of Syn4 and GST. Band intensities of Syn4 association with GST-VAMP2 were quantitated and the ratio of Syn4 to GST-VAMP2 determined (normalized to unstimulated GFP-Ad = 1 in each experiment); * $p < 0.05$, versus unstimulated GFP. **B)** Immunoblot to demonstrate the relative expression of GFP and GFP-39-70 proteins in the starting lysates used in GST-VAMP2 interaction assays. **C)** Basal PAK1 phosphorylation was evaluated by immunoblotting lysates prepared from transduced and unstimulated MIN6 cells. The marker indicates the upper phospho-PAK1 band; lower band is non-specific. Blots were subsequently stripped and reprobed for total PAK1 protein. Images are representative of three independent sets of transduced cell lysates.

3.3.4 Syntaxin 4-Gelsolin complexes and the K_{ATP} -channel-dependent/triggering pathway in MIN6 beta cells

Islets are composed of multiple cell types. To ascertain whether erratic short-term insulin secretion outcomes from islet studies were due to possible effects of non-insulin secreting cell types within islets, secretion studies were performed in the clonal MIN6 beta cell system. Mouse clonal MIN6 cells are considered to have similar insulin content and glucose-stimulatory response similar to that of normal islets, where normal responses occur at 16-20 mM glucose (244). As observed in mouse islets, expression of the GFP-39-70 but not the GFP-Scr peptide in MIN6 cells induced elevation of basal secretion in the absence of secretagogue (Figure 3-9A and 9B), though to a lesser extent than observed in whole islets. Similar to islets, prolonged glucose stimulation failed to unveil significant differences in insulin release between GFP and GFP-39-70 expressing cells (data not shown). By contrast, acute stimulation (10 min) with either glucose or KCl in GFP-39-70-expressing cells elicited small but significant ~20% attenuations of acute (10 min) stimulus induced secretion (Figure 9C): 20 mM glucose (205 ± 22 and 169 ± 20 ng/mg protein for GFP versus GFP-39-70, respectively), and with 35 mM KCl (499 ± 48 and 410 ± 24 ng/mg protein for GFP versus GFP-39-70, respectively). The combined impairments in basal and secretagogue-stimulated secretion culminated in significant losses in stimulation index (Figure 3-9D), and were not due to GFP-39-70-induced beta cell apoptosis, as evaluated by caspase-3 cleavage (Figure 3-9E).

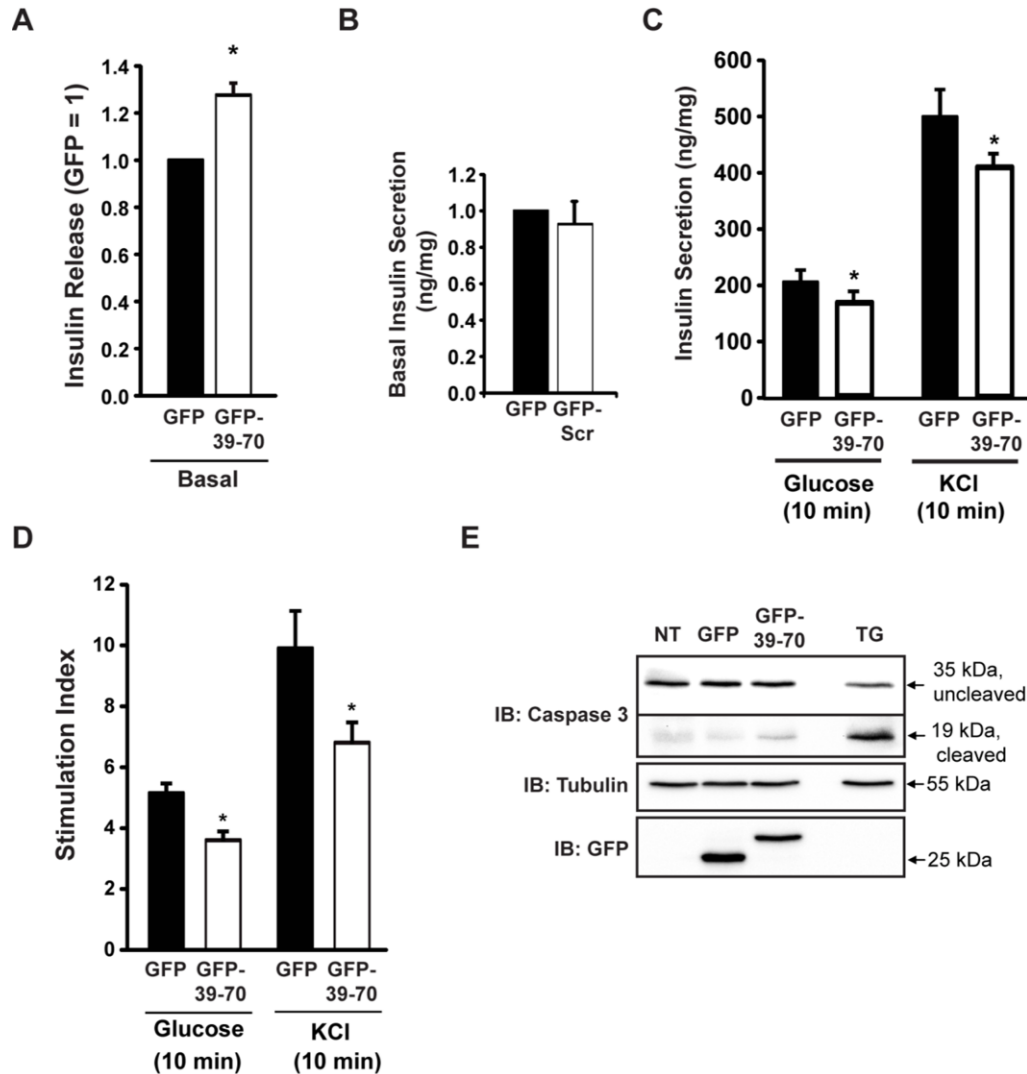


Figure 3-9. Disruption of Syn4-Gelsolin complexes impairs insulin secretion from MIN6 beta cells. **A)** MIN6 cells adenovirally transduced to express either GFP or GFP-39-70 proteins were pre-incubated in MKRBB for 2 h and left unstimulated to determine the insulin secretion stimulation index (SI = stimulated insulin release/basal insulin release). **B)** MIN6 cells electroporated to express the GFP-39-70 or the GFP-Scr peptide in MIN6 cells were evaluated for effects upon basal secretion. Data represent the average \pm S.D. of two independent sets of electroporated cells. **C)** MIN6 cells adenovirally transduced to express either GFP or GFP-39-70 were preincubated in MKRBB for 2 h and stimulated with 20 mM glucose or 35 mM KCl for only 10 min. Insulin secreted into the buffer was quantified by RIA and normalized for total protein content in corresponding cell lysates. Data represent the mean \pm S.E. of at least three independent experiments, * $p < 0.05$, versus GFP. **D)** Data from C represented as stimulation index. Data in C, and D represent the mean \pm S.E. of at least three independent islet batches; * $p < 0.05$, versus GFP. **E)** Caspase cleavage was assessed in MIN6 cells transduced to express GFP or GFP-39-70 by immunoblotting (IB) for caspase-3, which recognizes the 35 kDa inactive protein and the 19 kDa cleaved activated form. Treatment for 16 h with 0.5 μ M thapsigargin (TG) to induce apoptosis served as a positive control. The GFP immunoblot shows expression of GFP and GFP-39-70 (NT, not transduced) and tubulin immunoblot used as a loading control. Data are representative of two independent experiments.

Another method used to assess the phases of glucose-stimulation is by the diazoxide paradigm. This pharmacological paradigm is based upon the premise that the first phase release is due to ATP-sensitive potassium channel (K_{ATP})-dependent triggering, while the second phase is regulated by K_{ATP} channel-independent amplifying effects (2, 245-247). By subjecting MIN6 beta cells to depolarizing KCl concentrations in the presence of the K_{ATP} channel opener diazoxide, low glucose simulates triggering, and high glucose simulates amplification (44). Using this paradigm, GFP-39-70-expressing cells showed ~30% decrease in the KCl-stimulated triggering of insulin secretion compared to GFP-expressing controls (Figure 3-10A). Diazoxide action was verified by abolishment of secretion from cells treated with diazoxide alone or in combination with glucose in the absence of depolarizing KCl concentrations (data not shown). The GFP-expressing cells exhibited the amplifying response, with ~3-fold more insulin release upon stimulation with glucose, validating responsiveness of the cells (44, 47). However, the stimulation index of the amplification effect between GFP- and GFP-39-70-expressing cells was similar (Figure 3-10A, inset graph). This suggested that artificial disruption of the Syntaxin 4-Gelsolin complex by the GFP-39-70 peptide may have compromised triggering. Efforts to use this paradigm in adenovirally transduced islets failed however, as a combination of these islets and diazoxide treatment dramatically reduced insulin content (data not shown).

We next determined whether triggering steps impacted the Syntaxin 4-Gelsolin complex dissociation. The triggering pathway is defined as that involving closure of K_{ATP} channels, membrane depolarization, opening of voltage-dependent calcium channels, calcium influx and rise in $[Ca^{2+}]_c$, and activation of the exocytotic machinery (248). Testing these steps, acute KCl stimulation dissociated Syntaxin 4-Gelsolin complexes (Figure 3-10B). Consistent with this, treatment with nifedipine, which blocks calcium influx through the beta cell's voltage dependent calcium channels, blocked glucose-induced dissociation of endogenous Syntaxin 4-Gelsolin complexes (Figure 3-10C). However, Fura-2 calcium imaging experiments revealed no decrease in KCl-stimulated increases in $[Ca^{2+}]_c$ in GFP-39-70-Ad expressing cells versus GFP cells (Figure

3-10D), suggesting that the GFP-39-70 peptide does not disrupt Syntaxin 4-Gelsolin complexes and acute insulin secretion via disrupting calcium influx/elevation of $[Ca^{2+}]_c$ per se. Taken together with the abnormal activation of SNARE machinery (Syntaxin 4) induced by the GFP-39-70 peptide data of Figure 8, these data support the conclusion that a stimulatory defect induced by the GFP-39-70 peptide was downstream of calcium influx/elevated $[Ca^{2+}]_c$, perhaps at steps of Gelsolin activation and/or SNARE-mediated granule docking/fusion. Indeed, the calcium sensitivity of Gelsolin activation is ~300-1000 nM, consistent with that of insulin exocytosis (147, 249).

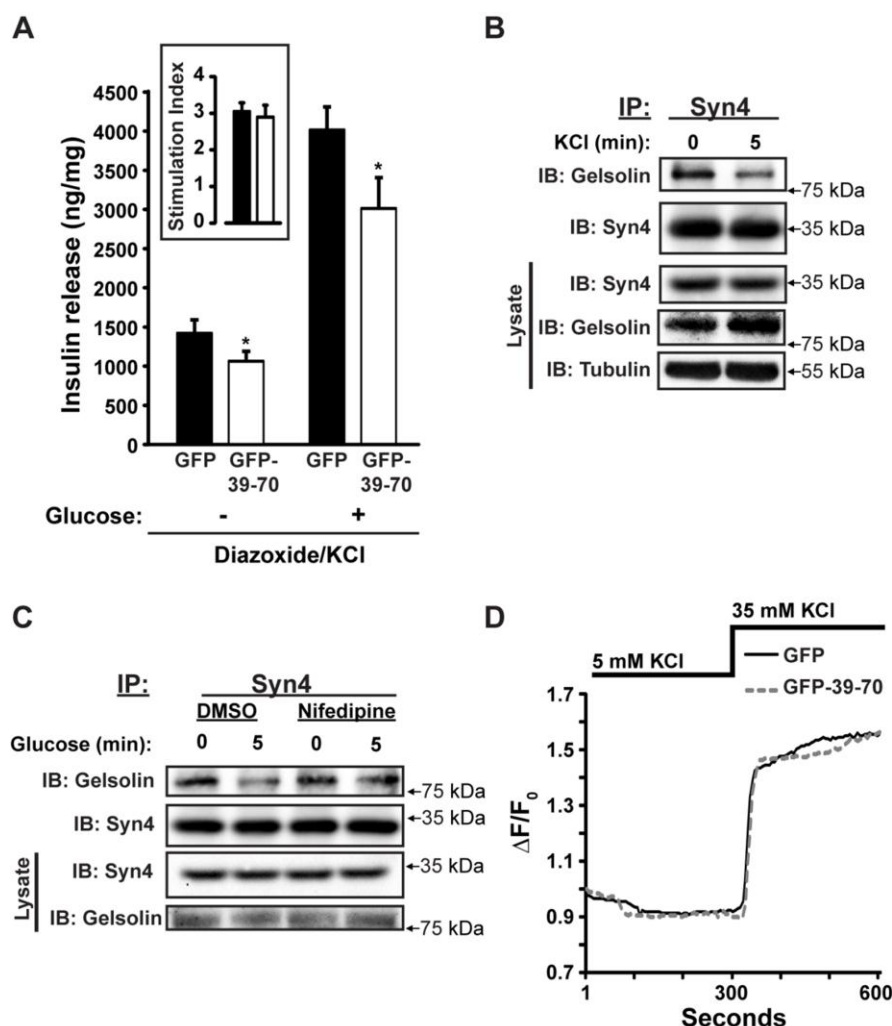


Figure 3-10. Disruption of Syn4-Gelsolin complexes impairs the triggering pathway. **A)** Effects of GFP-39-70 were evaluated by the diazoxide paradigm. MIN6 cells transduced to express GFP or GFP-39-70 proteins were incubated with 250 μ M diazoxide and 35 mM KCl, followed by addition of 20 mM glucose for 30 min and upon insulin secretion quantified. Insulin released was normalized for corresponding cellular protein content (ng insulin/mg protein). *Inset*, stimulation index = insulin release under diazoxide+KCl+glucose treatment / insulin release under diazoxide+KCl treatment alone (black bar=GFP, open bar=GFP-39-70). Data represent the mean \pm S.E. of at least three independent islet batches; * $p < 0.05$, versus GFP. **B)** MIN6 cells preincubated in MKRBB were stimulated 5 min with KCl (50 mM) and resultant cell lysates used for Syn4 IP as described in Figure 3-1B. Data are representative of five independent experiments. **C)** MIN6 cells treated with vehicle (DMSO) or nifedipine for 1 h during preincubation in MKRBB were acutely stimulated with glucose (20 mM) for 5 min, in the continued presence of nifedipine/DMSO, and resultant cell lysates used in anti-Syn4 immunoprecipitation reactions. Protein abundances in starting input lysates (100 μ g) were confirmed on a separate gel. Data are representative of three independent experiments. **D)** MIN6 cells on glass coverslips transduced to express GFP or GFP-39-70 proteins were pre-incubated in MKRBB supplemented with 2 mM glucose for 2 h followed by loading with Fura-2/AM. Cells were subjected to perfusion for 300 sec under low KCl (5 mM) conditions and switched to buffer containing stimulatory KCl (35 mM) for an additional 300 sec to evaluate calcium influx. Each 340/380 ratio trace was normalized to the initial basal value (F_0) of the respective trace to yield $\Delta F/F_0$.

3.4 DISCUSSION

In this report we demonstrate the existence of a novel interaction between the SNARE protein Syntaxin 4 and the F-actin-severing/capping protein Gelsolin, and that this interaction was susceptible to disruption upon glucose- and KCl-stimulation in beta cells. Introduction of a competitive inhibitory peptide, GFP-39-70, into pancreatic beta cells disrupted endogenous Syntaxin 4-Gelsolin complexes under basal conditions, mimicking the action of secretagogue-stimulation, providing strong evidence for a crucial functional role for this complex in clamping unsolicited basal insulin secretion. Mechanistically, disruption of Syntaxin 4-Gelsolin complexes spurred inappropriate activation of Syntaxin 4 under basal conditions, consistent with the concept of Gelsolin binding to Syntaxin 4 to mitigate unsolicited Syntaxin 4-mediated granule docking/fusion events. In addition to a role in the clamping mechanism, data gained from MIN6 cells suggests that Syntaxin 4-Gelsolin complexes may also participate in the calcium- and K_{ATP} -channel dependent/triggering mechanism of insulin release, although further studies will be required to substantiate this possibility in primary cells. Such a dual action of Syntaxin 4-Gelsolin complexes would be consistent with very recent work showing that actin filaments both prevent and augment the exocytosis of a single regulated secretory granule (250).

Gelsolin is a known calcium-activated F-actin severing/capping protein consisting of six homologous domains, each of which contain calcium binding sites that contribute to calcium-induced alterations to Gelsolin's conformation and activity (145, 147, 251, 252). Since Gelsolin is activated by calcium at levels observed in KCl- and glucose-stimulated beta cells (147, 249), it could be speculated that Gelsolin participates within calcium microdomains for F-actin clearance at active sites of exocytosis. If so, one explanation for the effect of the GFP-39-70 peptide is that it displaced Syntaxin 4 from a calcium microdomain. However, Syntaxin 4 remains at the PM in cells treated with latrunculin B, and moreover, that Syntaxin 4 is activated (64) and presumed to be functional given the potentiating effect of latrunculin upon calcium-stimulated insulin release

(33). Regarding Gelsolin's role at calcium microdomains for actin clearance, this remains controversial: Gelsolin knockdown in the MIN6B1 cell line ablated glucose-induced F-actin remodeling, but the recent analyses of islets from Gelsolin knockout mice do not show effects upon F-actin remodeling (151), and now we also show that the GFP-39-70 peptide fails to impact actin remodeling. The lack of effect of the GFP-39-70 peptide upon actin remodeling under basal conditions could be explained by the need for calcium activation of Gelsolin, and that its mere dissociation from Syntaxin 4 was insufficient to induce actin remodeling. Moreover, the absence of a refractory response from the cytoskeleton to the competitive peptide, could suggest that Gelsolin's severing activity was unaffected by the peptide. As such, the GFP-39-70 peptide would likely not induce the many and diverse defects associated with Gelsolin knockout or knockdown, such as compensatory increases in Rac1 protein, reduced fibroblast motility, neurological and immune system defects (98, 243, 253), and did not cause apoptosis as was observed in Gelsolin-depleted MIN6B1 cells (152). Because the degree of change in stimulated insulin secretion was similar in cells depleted of Gelsolin (36%) and those expressing the GFP-39-70 peptide (~30%), the implication is that Gelsolin's mechanism of action in glucose-stimulated insulin secretion might be through its interaction with Syntaxin 4. This may be further impacted by phosphoinositide binding and regulation of Gelsolin (254-256). The lack of a larger deficit in insulin secretion could be due to compensation by a closely related family member, Scinderin, which shows expression only in adrenal chromaffin cells, kidney and intestinal cells (154), and pancreatic beta cells (data not shown). Scinderin and Gelsolin share more than 60% sequence identity, and a Scinderin-derived actin-binding peptide inhibited Ca^{2+} -dependent exocytosis without affecting the whole-cell Ca^{2+} current, by 61% in mouse pancreatic beta-cells (159). Thus, the potential remains that Gelsolin and Scinderin work in an additive manner, perhaps via Syntaxin 4; due to Scinderin's more limited expression profile, Syntaxin 4-Scinderin complexes in the beta cell could present a means to selectively manipulate insulin release.

Poorly regulated/constitutive and non-secretagogue specific insulin release from an islet beta cell is characteristic of an aberration of the regulated exocytosis (SNARE protein) machinery; F-actin, which is shown to function both in preventing unsolicited basal exocytosis and in promoting secretagogue-induced exocytosis in endothelial cells (250), may also be defective. Syntaxin 4 activation appears to be linked to its association with F-actin, which vacillates in the presence of secretagogue (64). As such, one possible reason that the GFP-39-70 peptide disrupted both basal and stimulated secretion in the MIN6 cells is that it may have interfered with normal activation/deactivation cycling of Syntaxin 4: following fusion, SNARE complexes undergo disassembly with t-SNARE proteins deactivated and recycled for subsequent rounds of docking/fusion. Furthermore, residues within the t- and v-SNARE proteins that become surface exposed upon conformational changes induced by assembly into the heterotrimeric SNARE core complex have been linked directly to calcium triggering of exocytosis from chromaffin cells (257). Since Syntaxin 1A can associate directly with the operative L-type voltage dependent calcium channels in beta cells (258, 259), placing SNARE core complex formation at the site of calcium entry into the cell, future studies of Syntaxin 4 binding to channels will be required to determine if this could also account for GFP-39-70 peptide induced defects. Alternatively, constitutive Syntaxin 4 activation induced by the peptide could have disrupted binding and function of SNARE accessory proteins that bind calcium, such as Synaptotagmin 7 and Doc2b, both of which function in insulin secretion (201, 260-262). Nevertheless, despite our utilization of multiple and diverse approaches, we were unable to reproducibly detect defects in first phase/triggering in GFP-39-70 expressing islets. One possibility is that the peptide exerted effects in non-beta cells of the islet, particularly since Syntaxin 4 and Gelsolin are considered ubiquitously expressed proteins, and this generated sufficient 'noise' in our systems to negate detection of the small expected changes in secretion. De-regulation of basal secretion in the islets was also far more substantial (4-fold) than in MIN6 beta cells, effectively blunting any further secretagogue-induced increase in stimulation index. Notably however, actin filaments prevent

hypersecretion as well as promote stimulated secretion (250). Given that changes in actin tethering to Syntaxin 4 occur in tandem with Gelsolin, it remains possible that GFP-39-70 has impacted this process. Future studies utilizing real-time imaging of in-cell Syntaxin biosensors that distinguish open from closed conformations will be required to determine its cyclic activation patterns in primary islet beta cells.

The Ha domain of Syntaxin 4, in isolation, has not previously been shown to be sufficient to confer its binding to any other of Syntaxin 4's binding partners other than Gelsolin. Syntaxin 4 directly interacts with both Gelsolin and F-actin, and Gelsolin directly binds to F-actin, however it remains unknown whether the three form a heterotrimeric complex. The simultaneous dissociation of Syntaxin 4 from F-actin and from Gelsolin in response to acute glucose stimulation in beta cells might support this concept, although efforts to reciprocally co-immunoprecipitate the complex from cell lysates, or efforts to capture the complex *in vitro*, were unsuccessful due to technical limitations with available reagents. As such, the inappropriate Syntaxin 4 activation could potentially be caused by the dissociation of F-actin that happens alongside disruption of the Syntaxin 4-Gelsolin complex. Indeed, this is seen using a peptide containing the minimal region of Syntaxin 4 that binds F-actin (GFP-39-112), or through global F-actin depolymerization using the actin monomer binding agent Latrunculin B (64). Oddly, treatment with Latrunculin failed to also elevate basal insulin secretion, suggesting that there is distinction between non-specifically disrupting the entire F-actin cytoskeleton and disrupting select protein-protein interactions, as in the case of using GFP-39-70 to disrupt Syntaxin 4-Gelsolin complexes. The idea that basal insulin secretion is influenced by the cytoskeleton is supported by studies of EphA-Ephrin-A signaling, cell-cell contact and Focal Adhesion Kinase (132, 136, 239).

It is interesting that cells expressing GFP-39-70 and further subjected to acute glucose stimulation did not elicit an additive effect upon disruption of Syntaxin 4-Gelsolin complexes, such that a contingent of complexes persisted. This phenomenon was also observed with peptide

disruption of F-actin-Syntaxin 4 binding (64). One possibility is that the peptide is only capable of mimicking the action of secretagogue and only disrupts a subset of complexes; some may be refractory or inaccessible. Furthermore, it remains unknown as to whether Syntaxin 4 associates, transiently or otherwise, with Gelsolin and/or F-actin during the dynamic process of SNARE complex assembly/disassembly; dissociation events may be so transient that it is not possible to detect all dissociated in sync. Although it also remains possible that the peptide is a relatively poor competitor, despite our lysate evidence showing it to be abundantly expressed, future studies of Syntaxin 4-Gelsolin complex kinetics are required to address issues related to dissociation susceptibility.

In conclusion, we describe the first mechanistic evidence for Gelsolin in regulating the exocytosis machinery, via its direct association with the t-SNARE protein Syntaxin 4. Through association with Syntaxin 4, Gelsolin plays a critical role as a clamp on unsolicited Syntaxin 4 activation and aberrant insulin release in the absence of the appropriate secretagogue signal. Calcium may act as the key which unlocks the Gelsolin clamp, as it is known to initiate Gelsolin conformational changes. Once released from Gelsolin, Syntaxin 4 activates and joins other SNARE proteins to facilitate granule docking and fusion. Importantly, elevated basal insulin release observed in islets with peptide-induced disruption of Syntaxin 4-Gelsolin complexes models the constitutive insulin release observed in pre-diabetic and type 2 diabetic patients, prior to onset of beta-cell apoptosis; this may provide new insight into the dysregulation of insulin exocytosis in diabetes. From a broader perspective, Gelsolin and Syntaxin 4 are ubiquitously expressed proteins, such that their interaction may represent a more generalized clamping mechanism required for maintaining the ‘regulated’ aspect of SNARE-mediated exocytosis.

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CHAPTER 4. CONCLUDING REMARKS

The objective of this dissertation was to elucidate mechanisms of F-actin remodeling and SNARE-mediated insulin secretion in pancreatic beta cells. My dissertation work encompasses the initial glucose-induced activation of the Cdc42-PAK1 pathway which leads to ERK activation, the F-actin remodeling process, and finally the effects of glucose and the Cdc42-PAK1 pathway upon Syntaxin 4 during the final steps of insulin exocytosis. Here, I will discuss the primary issues addressed in each chapter of my dissertation, including how this work impacts the field of beta cell biology and other systems, with suggestions towards additional work needed in those specific areas. Additionally, I propose future studies pertaining to F-actin remodeling, signal transduction, and SNARE protein regulation in the beta cell. These studies will be required to progress our understanding regarding the mechanisms regulating insulin secretion.

The role of PAK1 in secretory processes is a largely understudied area. PAK1 is classically known to be involved in cell survival and motility and is an actively researched target for anti-cancer therapies (73, 263, 264). PAK1 has also been hypothesized to be a bridge between GTPase signaling and the MAP kinase pathways (87, 175). Our lab has shown that PAK1 is critical during the second phase of insulin secretion from human and mouse pancreatic islets and in insulin-stimulated GLUT4 translocation in mouse skeletal muscle (43, 45). My dissertation work supports these observations in beta cells and extends our knowledge beyond Cdc42 to show that PAK1 signaling 1) facilitates glucose-induced cortical F-actin remodeling through MEK-ERK signaling, 2) mediates MEK-ERK signaling that is important for amplifying insulin secretion, 3) is required for the accumulation of VAMP2-bound insulin granules at the PM, and 4) regulates Syntaxin-4-F-actin complexes. I found that PAK1 activation is required for MEK phosphorylation in beta cells, which is consistent with reports of PAK1-MEK signaling *in vitro* and in rodent fibroblast cell lines and HEK293 cells and explains why the PAK1 KO mouse islets had defective glucose-induced ERK activation (45). Additionally, PAK1 and ERK activation are required for glucose-induced F-actin remodeling, which plays an important role regulating glucose-induced insulin secretion. Indeed, blocking PAK1 activation attenuates a very

distal event in the process of insulin granule exocytosis, the transit/retention of VAMP2-bound insulin granules at the plasma membrane. These data contribute to our understanding of why PAK1 inhibition selectively reduces the amplification/second phase of glucose-stimulated insulin secretion, the phase which is more dependent on granule mobilization. Finally, PAK1 inhibition disrupts Syntaxin 4-F-actin complexes in the basal state without increasing basal insulin secretion, which links PAK1 signaling to SNARE-mediated exocytosis for the first time. It is noteworthy that other molecules can also act through PAK1, and Cdc42 may not be the sole upstream initiator of PAK1 activity. For example, recent work suggests that the SAD-A protein kinase can activate PAK1 in beta cells (224). SAD-A has also been implicated in activating cAMP-potentiated glucose-stimulated insulin secretion dependent on PAK1, possibly placing PAK1 downstream of PKA-dependent signaling.

The knowledge gained from my studies of PAK1 signaling in the beta cell has significant potential to influence research in other fields. PAK1 signaling is implicated in insulin-stimulated GLUT4 translocation (45), GLP-1 secretion from enteroendocrine L cells (229), and neuronal physiology (265). As for islet cell types other than beta cells (alpha cells, delta cells, PP cells), investigation into the role of PAK1 in alpha cells is still in its infancy (45). Glucagon secretion is normal in islets from PAK1 knockout mice (45), but further studies in metabolically stressed animals (e.g. high-fat diet) or stressed islets *ex vivo* (e.g. high glucose/palmitate) will be required to determine whether PAK1 has any role in alpha cells. In fact, MEK inhibitors can block ghrelin-induced glucagon secretion and ERK phosphorylation in the alpha cell line, alpha-TC1 (266). My data point to new and previously unrecognized PAK1 signaling pathways and can serve as rationale for considering unorthodox roles for PAK1 in other secretory cell types.

Back in 2006 it was reported that Gelsolin was involved in glucose-stimulated insulin secretion, although the exact mechanisms for this were undefined (96). My work has added to the understanding of Gelsolin function in beta cells. I showed that Gelsolin interacts with the SNARE protein Syntaxin 4, a key regulator of biphasic insulin secretion (39, 143), and that the

ability of glucose to target this association in a dynamic manner is important to maintaining normal low basal secretion as well as normal glucose stimulated biphasic secretion. Syntaxin 4 directly interacts with F-actin and, in response to glucose, Syntaxin 4-F-actin complexes dissociate (64, 143). Disruption of Syntaxin 4-F-actin with a competing peptide or through depolymerization of F-actin with latrunculin leads to increased Syntaxin 4 activation and insulin secretion, but it was unclear how this occurs (64). My findings suggest that the binding of Gelsolin to Syntaxin 4 regulates Syntaxin 4-F-actin interaction and Syntaxin 4 activation. PAK1 also regulates Syntaxin 4-F-actin complexes, but this may not be the case for Syntaxin 4-Gelsolin complexes. Syntaxin 4-Gelsolin complexes respond to calcium influx alone, while calcium influx alone cannot activate Cdc42 (43). It could be speculated that PAK1 activation of Rac1 might inactivate Gelsolin at later time points of glucose stimulation (20-30 min). The possibility remains that other F-actin binding proteins also bind to Syntaxin 4 to bridge indirect F-actin association, such as alpha-fodrin (160, 161). In addition, the closest relative of Gelsolin, Scinderin (also called Adseverin), interacts with Syntaxin 4 and may play a role similar to Gelsolin in the regulation of Syntaxin 4 and insulin secretion (Kalwat and Thurmond, unpublished data). A single study exists on Scinderin in primary beta cells, in which small interfering peptide regions of Scinderin were used to block its activity in patch-clamp experiments (159). In that study, blocking Scinderin activity prevents Ca^{2+} - and GTP-stimulated secretion (159) although further studies are required to validate the effects of more specific inhibition of Scinderin via knockdown. Since Gelsolin and Scinderin have different calcium sensitivities, their interaction with Syntaxin 4 may underlie differential responses to varying magnitudes of stimulation (158). The precise mechanisms underlying Gelsolin regulation of Syntaxin 4 activation are unclear. However, since I found that Ca^{2+} influx is both necessary and sufficient for Syntaxin 4-Gelsolin dissociation, it is likely that Ca^{2+} signaling is involved in Syntaxin 4 activation. Gelsolin is a Ca^{2+} -activated F-actin severing protein, therefore investigations using Ca^{2+} -binding mutants of Gelsolin (267) will be required to determine

whether the effect of Ca^{2+} on Syntaxin 4-Gelsolin is direct or indirect. My work also pinpointed the region of Syntaxin 4 which is sufficient to confer Syntaxin 4 binding to Gelsolin, the Syntaxin 4 39-70 residue region (Ha helix). This is a novel interaction to date, although it suggests that other syntaxin isoforms may have an unrecognized potential to interact with F-actin binding proteins in a manner similar to Gelsolin via this region.

4.1 FUTURE STUDIES

4.1.1 The Cdc42-PAK1 Pathway

Small Rho GTPases have been implicated in beta cell biology for the last 15 years (69), and even though much research has been done, we still lack knowledge concerning many key events which occur in the Cdc42-PAK1-Rac1 pathway in response to glucose. Such events include the identity of the glucose-specific signal that activates Cdc42, the exact effectors of PAK1 signaling in the beta cell, and how this pathway links to exocytosis.

It is clear that the metabolism of glucose is required for Cdc42 activation, as non-metabolizable analogs do not cause activation (43). Studies using various metabolites that can enter certain steps of glycolysis (i.e. glyceraldehyde) or the citric acid cycle (i.e. methylpyruvate) will be required to determine the exact source of the metabolite(s) which initiate Cdc42 activation. It would not be surprising if these metabolites activated certain GEFs for Cdc42 (β Pix) or deactivated certain GDIs (RhoGDI and Cav-1) and GAPs. As we have recently found, β Pix is a GEF for Cdc42 in the beta cell and is required for proper Cdc42 activation in response to glucose (74), but this does not preclude the existence of additional GEFs that may play a role. It has also been found in beta cells during time points preceding Cdc42 activation at 3 min, that the Arf6 and ARNO proteins are activated and are required for Cdc42 activation, although the Arf6/ARNO pathway is not glucose-specific (75). Therefore the identity of the glucose-specific Cdc42 activator is still unclear.

The recent publication suggesting that SAD-A kinase is an upstream activator of PAK1 (224) brings up the question of whether or not SAD-A activation is dependent on Cdc42 activation. The involvement of Cdc42 could be tested using either siRNA-mediated depletion of Cdc42 or through the use of a novel Cdc42 small molecule inhibitor called ML-141. After ablation of Cdc42 function in beta cells, SAD-A kinase activation could be assessed by immunoblot. This would be supported by more detailed studies of the time course of SAD-A kinase activation in response to stimuli, since a Cdc42-dependent process would be expected to occur after 3 min of glucose stimulation. Preliminary data from our own laboratory suggests PAK1 also to be downstream of cAMP-potentiated secretion, since IPA3 treatment disrupts GLP-1 potentiated secretion, and GLP-1 signaling is known to involve cAMP and PKA signaling (Zhanxiang Wang, unpublished data). If Cdc42-PAK1 is indeed part of the GLP-1/cAMP signaling pathway, it would be pertinent to test whether this is through PKA, Epac2, or both using analogs of cAMP that specifically activate either PKA or Epac2.

Linkage between the Cdc42-PAK1 pathway and SNARE-mediated insulin secretion may not be an improbable idea. Indeed, Cdc42 interacts with VAMP-2 and can participate in a tertiary complex with VAMP-2 and Syntaxin 1A (86). Additionally, since PAK1 is capable of localizing to membranes and can phosphorylate multiple substrates that regulate F-actin dynamics, PAK1 may regulate F-actin interaction with proteins on the plasma membrane (73). This notion is supported by my results showing that blocking PAK1 activation with IPA3 disrupts the normal Syntaxin 4-F-actin interaction (Figure 2-6). This suggests that PAK1 could indirectly affect Syntaxin 4-F-actin complexes through its influence on F-actin dynamics.

4.1.2 The ERK Pathway

If or how the ERK pathway functions in glucose-stimulated insulin secretion is controversial. Whether ERK plays any acute signaling roles in insulin secretion and/or whether it solely mediates its effects through transcriptional activation of key beta cell genes is the crux of

the controversy. It is clear that ERK is activated shortly after stimulation with secretagogues or growth factors and that ERK can subsequently translocate to the nucleus to mediate effects on transcription (178, 223). A sizeable portion of active ERK remains in the cytosol as well (178), which raises the question about what cytosolic substrates ERK may act on in beta cells. Recently, a screen in NIH 3T3-L1 fibroblasts yielded 67 novel ERK2 substrates (268), including two Cdc42 effector proteins (CDC42EP1 and 2) and myosin IXb. In another study, ERK was shown to activate MLCK (171). Since many ERK substrates are linked to F-actin dynamics, future investigations should assess whether these substrates have roles in the beta cell. Discrepancies between studies regarding the impact of ERK inhibition on insulin secretion may stem from differences in cell lines, islet sources, and the duration and concentrations of glucose and/or MEK-specific inhibitors used. These variations lead to cases where inhibition of ERK activation either showed no effect on secretion (178), versus where it depresses secretion but to differing degrees of severity (96, 180). Further, conclusions based upon knockdown of ERK levels in beta cells must be interpreted carefully as knockdown usually requires 48 hours, leaving open the possibility for transcriptional changes (95). In fact, inhibition of ERK for 36 hours was shown to prevent glucose-induced insulin gene transcription in MIN6 cells (269). Future work will require short-term (1-2hr) MEK inhibition to ascertain the need for acute glucose-dependent ERK activation in Syntaxin 4-F-actin dynamics and SNARE complex formation.

4.1.3 Regulation of F-actin Dynamics

There is a paucity of knowledge about F-actin binding proteins downstream of Cdc42 or PAK1 that may be important in glucose-stimulated F-actin remodeling and insulin secretion. In addition to Gelsolin, its closely related family member, Scinderin, may have a role in the beta cell. Since Scinderin has a critical role distinct from Gelsolin in adrenal chromaffin cell secretion (126, 270) and is capable of binding to Syntaxin 4 in beta cells, the possibility exists that Scinderin is a regulator of insulin secretion and SNARE-F-actin association. The use of

knockdown approaches to reduce levels Scinderin alone or both Scinderin and Gelsolin will likely be required to determine the relative contribution of Scinderin to insulin secretion and glucose-induced F-actin remodeling in beta cells. Scinderin would be an intriguing target for pharmacological intervention of insulin secretion since its expression is limited to neuroendocrine and intestinal cells as opposed to Gelsolin which is ubiquitously expressed (154, 156).

While it is true that Profilin and Arp2/3 are potential candidates for glucose-induced F-actin remodeling, during my studies I chose to first investigate the Cdc42-PAK1 pathway and potential downstream PAK1 targets. The evidence that PAK1 is both crucial for second phase insulin secretion and downstream of Cdc42 points supports this line of research. While Arp2/3 is downstream of Cdc42, it is also possible that PAK1 can signal to Arp2/3 (93). In light of my recent data regarding the ~30% deficit in GSIS from MIN6 cells where ERK activation is completely blocked, it makes sense to investigate alternative PAK1 signaling pathways that may account for the remainder of GSIS not accounted for by PAK1-MEK-ERK signaling.

With regard to Profilin, not much information is available pertaining to its role in stimulated exocytosis, but it has been shown to have a regulatory role in synaptic vesicle exocytosis through actin polymerization (271). In eukaryotic cells, Profilin promotes the polymerization of actin by binding G-actin and promoting exchange of ADP for ATP (144) to ultimately deliver actin monomers to the barbed end of growing actin filaments. Inactivation of Profilin can be mediated via its binding to PIP and PIP₂, and in this way its regulation may be linked to membrane processes (144). Maintenance of an intact cortical F-actin cytoskeleton in the basal state is critical for regulation of stimulated insulin secretion, since treatment of islets with latrunculin causes massively unregulated secretion in response to glucose while leaving basal secretion unaffected (33, 64, 130). In some instances, glucose has been shown to elicit the net polymerization of F-actin in islets, implicating a need for regulatory proteins like Profilin (272). Profilin interacts with Arp2 of the Arp2/3 complex and has been suggested to interact with WASP proteins (144), and both Arp2/3 and WASP are currently being investigated for a

role in beta cell F-actin remodeling (85). Studies of proteins like Profilin will thus be required to expand our understanding of how glucose-induced F-actin remodeling is mediated and how it ultimately regulates insulin granule exocytosis.

Of the Rho family GTPases, Cdc42 and Rac1 were shown to be the major forms operative in GSIS (69); RhoA inhibition was less impactful upon GSIS in the hamster insulinoma cell line HIT. Quite unexpectedly, during the course of my studies on Gelsolin and PAK1 in GSIS, another group demonstrated that RhoA and one of its key effectors Rho-associated kinase (ROCK) did indeed impact GSIS (70, 273), but as negative regulators of insulin secretion, as their pharmacological inhibition enhanced insulin secretion in sorted primary rat beta cells (70). The discrepancies between these findings could have to do with primary cell versus cell line differences and in the duration of treatment with the RhoA inhibitor (24 h in the former, 48 h in the latter). Studies in secretion from neutrophils show that RhoA has a role on the secretory granule to block localized actin depolymerization or alternatively facilitate F-actin polymerization (99, 274). Since ROCK is known to cause MLC activation through inhibiting myosin light chain phosphatase (275) and since RhoA signaling and Cdc42/Rac1 signaling have been reported to oppose each other (276-278), inhibition of the RhoA-ROCK pathway may be worthy of pursuit for new therapies for type 2 diabetes. Indeed, multiple inhibitors of ROCK have been developed and seem to be beneficial in beta cell lines (279-281), although the potential effects on the beta cell *in vivo* remain to be determined. In addition, the opposing roles of RhoA and Cdc42/Rac1 need to be verified in beta cells, therefore future studies are required to fully understand how RhoA might intertwine with the Cdc42-PAK1 pathway and F-actin remodeling in beta cells.

4.1.4 Regulation of Syntaxin 4

Exactly how Syntaxin 4 is activated to form the tertiary SNARE complex and ultimately insulin exocytosis is not understood. Currently, evidence suggests a role for F-actin (64), Gelsolin (143), Munc18c O-linked glycosylation and tyrosine phosphorylation (202, 282), Doc2b

(202), and S-nitrosylation in the activation of Syntaxin 4 (210). There are other candidates, in particular Munc13-4, which may also regulate Syntaxin 4 activity. Munc13-4 was recently found to interact with the H3 domains of Syntaxins 1 and 4 *in vitro* in a Ca^{2+} -dependent manner (203). My results showed that Syntaxin 4-Gelsolin complex dissociation is dependent upon Ca^{2+} influx, but the mechanism behind this is unclear. It is known that Ca^{2+} binding to Gelsolin causes a conformational change which allows Gelsolin to sever F-actin filaments (147). Fortunately, a Ca^{2+} -binding deficient mutant of Gelsolin exists and future studies using this mutant can help to elucidate the molecular mechanism of Syntaxin 4-Gelsolin dissociation (267). Given that Syntaxin 1 and 4 have different roles in glucose-stimulated insulin secretion, it may be useful in the future to use bioinformatics analysis and mutagenesis to determine the residues which allow Syntaxin 4 to promote both phases of secretion and which restrict Syntaxin 1 to first phase. Additional studies might involve an analysis of differential binding proteins that exclusively regulate either Syntaxin 1 or 4. Examples of this are already known, especially in the case of the Munc18-1-Syntaxin 1 and Munc18c-Syntaxin 4 complexes (198, 283). Interestingly, there seems to be linkage between Syntaxin 4-Munc18c and Syntaxin 4-F-actin, as disruption of F-actin with latrunculin disrupts Syntaxin 4-Munc18c complexes and Munc18c knockdown disrupts Syntaxin 4-F-actin complexes (Oh and Thurmond, unpublished). The implications of these effects on cortical F-actin remodeling and insulin secretion are currently under investigation. Now that PAK1 is implicated as a potential regulator of Syntaxin 4-F-actin complexes future work will be required to determine whether ERK or another PAK1 effector is responsible for PAK1 regulation of Syntaxin-4-F-actin complexes. My data point to the regulation of Syntaxin 4 activation being a complicated multifaceted event not solely regulated by just one protein.

4.2 CONCLUSION

Altogether, these findings expand our knowledge of the control of glucose-stimulated insulin secretion by the Cdc42-PAK1 signaling pathway and the Syntaxin 4-Gelsolin interaction. I have expanded the known signaling cascade supporting second phase GSIS by elucidating PAK1-MEK-ERK signaling and revealed this to be important to actin remodeling. I have provided the first links of glucose-regulated Cdc42-PAK1 signaling to Syntaxin 4-F-actin dynamics. This marks the first signaling cascade in the beta cell to be required for F-actin remodeling, insulin granule accumulation at the PM, and SNARE complexation. These new interactions and linkages provide new facets of GSIS to target for therapeutic gain.

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CURRICULUM VITAE

Michael Andrew Kalwat

Education

- PhD** Indiana University September 2012
Department of Biochemistry and Molecular Biology
Indianapolis, IN
Cumulative GPA: 3.93/4.00
Dissertation Title: F-actin Regulation of SNARE-Mediated Insulin Secretion
Advisor: Debbie C Thurmond, PhD
- BS** Purdue University 2003 to 2007
Department of Biochemistry
West Lafayette, IN
Minor in Biological Sciences
Cumulative GPA: 3.47/4.00

Research Experience

- Graduate Research** 2008 to 2012
Indiana University School of Medicine, Indianapolis, IN
Advisor: Dr. Debbie C Thurmond
- Currently investigating glucose-stimulated signaling events in the pancreatic beta cell that result in F-actin remodeling and subsequent biphasic insulin secretion. Also exploring pathways involved in glucose-specific Cdc42 activation and SNARE-mediated exocytosis.
- Undergraduate Research** 2006 to 2007
Purdue University, West Lafayette, IN
Advisor: Dr. James D Forney, Department of Biochemistry
- Analyzed DNA Polymerases in Paramecium tetraurelia using RNA interference
- Undergraduate Research** 2005
Purdue University, West Lafayette, IN
Advisor: Dr. Ching-jer Chang, Department of Medicinal Chemistry and Molecular Pharmacology
- Assisted in thin-layer chromatography of potential anti-cancer agents

Funding

- American Heart Association Predoctoral Fellowship 2010 to 2012
- Educational Enhancement Travel Grant 2010
- Graduate Student Office Travel Fellowship 2010

Diabetes and Obesity Research Training Grant (T32)

2008 to 2010

Honors and Awards

Midwest Islet Club 2012 Oral Presentation Award (3rd Place)

2012

Indiana University School of Medicine Graduate
Student Mentor Program

2008 to 2012

University Fellowship, Indiana University School of Medicine

2007

Agricultural Research Fund Scholarship

2007

- Awarded during undergraduate research

Semester Honors

2004, 2006, 2007

Publications

Journal Publications

Ramalingam LR, Oh E, Yoder SM, **Kalwat MA**, Groffen AJ, Thurmond DC. Doc2b is Essential for Glucose Homeostasis *in vivo* via its Munc18c-sequestering action. *Diabetes* 2012. June 14 Epub ahead of print.

Oh E, **Kalwat MA**, Kim M-J, Verhage M, Thurmond DC. Munc18-1 regulates first-phase insulin release by promoting granule docking to multiple Syntaxin isoforms. *J Biol Chem*. 2012. Jul 27;287(31):25821-33.

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Kepner EM, Yoder SM, Oh E, **Kalwat MA**, Wang Z, Quilliam LA, Thurmond DC. Cool-1/ β PIX functions as a guanine nucleotide exchange factor in the cycling of Cdc42 to regulate insulin secretion. *Am J Physiol Endocrinol Metab*. 2011 Dec;301(6):E1072-80.

Wiseman DA, **Kalwat MA**, Thurmond DC. Stimulus-induced S-nitrosylation of Syntaxin 4 impacts insulin granule exocytosis. *J Biol Chem*. 2011 May 6;286(18):16344-54.

Jewell JL, Oh E, Ramalingam L, **Kalwat MA**, Tagliabracci VS, Tackett L, Elmendorf JS, Thurmond DC. Munc18c phosphorylation by the insulin receptor links cell signaling directly to SNARE exocytosis. *J Cell Biol*. 2011 Apr 4;193(1):185-99.

Journal Papers in Preparation

Kalwat MA, Wang Z, Yoder SM, Thurmond DC. Glucose Mediates Effects on F-Actin Remodeling and Amplification of Insulin Secretion in Pancreatic Beta Cells via PAK1-MEK-ERK Signaling. *In preparation*.

Presentations

Kalwat MA. A Novel P21-Activated Kinase (PAK1) Signaling Cascade Couples Glucose-Induced Amplification of Insulin Secretion to F-Actin Remodeling. Midwest Islet Club Conference. May 23-24, 2012.

Kalwat MA. Syntaxin 4-gelsolin complexes have a positive role in regulated insulin secretion. Center for Diabetes Research Seminar Series. May 19, 2011.

Kalwat MA and Thurmond DC. Gelsolin interacts with Syntaxin 4 to regulate insulin secretion. Midwest Islet Club Conference. May 27-29, 2009.

Posters

Kalwat MA, Wang ZW, Yoder SM, Thurmond DC. A Novel p21-Activated Kinase (PAK1) Signaling Cascade Couples Glucose-Induced Amplification of Insulin Secretion to F-Actin Remodeling. American Diabetes Association 72nd Scientific Sessions, Philadelphia, PA. June 8-12, 2012.

Kalwat MA, Wiseman DA, Luo W and Thurmond DC. Gelsolin Functions in Glucose-Stimulated Insulin Secretion through Association with Syntaxin 4. American Diabetes Association 71st Scientific Sessions, San Diego, CA. June 24-28, 2011. *Selected for Guided Audio Tour.*

Kepner EM, Yoder SM, **Kalwat MA,** Wang Z, Thurmond DC. Cool-1/ β Pix functions as a GEF protein in the cycling of Cdc42 to regulate insulin secretion. Midwest Islet Club, Madison, WI. May 25, 2011.

Wiseman DA, **Kalwat MA,** Thurmond DC. Stimulus-Induced S-Nitrosylation of Syntaxin 4 Impacts Insulin Granule Exocytosis. American Diabetes Association 71st Scientific Sessions, San Diego, CA. June 24-28, 2011. *Selected for Guided Audio Tour.*

Wiseman DA, **Kalwat MA,** Thurmond DC. Glucose-Induced S-Nitrosylation of Syntaxin 4 Regulates Insulin Exocytosis from Pancreatic β -cells. Midwest Islet Club, Indianapolis, IN. May 26-27, 2010.

Kalwat MA, Luo W and Thurmond DC. Syntaxin 4-Gelsolin Complexes Regulate Glucose-Stimulated Insulin Secretion. Midwest Islet Club, Indianapolis, IN. May 26-27, 2010.

Kalwat MA, Luo W and Thurmond DC. Syntaxin 4-Gelsolin Complexes Regulate Glucose-Stimulated Insulin Secretion. Keystone Symposia on Islet Biology, Whistler, BC, Canada. April 12-17, 2010.

Kalwat MA, Forney JD. RNAi Analysis of DNA polymerases in Paramecium tetraurelia. Purdue Undergraduate Poster Symposium, West Lafayette, IN. 2007.

Service

Biochemistry and Molecular Biology Student Representative

- Elected by the students to help organize departmental events and attended faculty meetings.
Served Fall 2010-Fall 2011.

JDRF Walk to Cure Diabetes

- Participant. Military Park, Indianapolis, IN. 2010 and 2011.